

Opinions differ on spending NIH gold

Differences within the US research community over how best to support biomedical research create the impression of factional quarrelling. That cannot be avoided, but it would help if there were a director of the NIH in post.

THE annual budget of the US National Institutes of Health (NIH), now more than \$8,000 million a year, is the largest single source of support for basic research in the whole wide world. This pot of gold, steadily enlarged over four decades, has created and sustained the US biomedical research community, which is the most productive research community anywhere. Yet there are now sharp divisions of opinion among researchers about the way in which the NIH budget is spent. One issue, raised by a report from the Institute of Medicine last month, is whether more should go on infrastructure (buildings and equipment) and on training in research. Another, not as clearly articulated, is that of the correct balance between research aimed at the improvement of known therapies and the search for radically new methods of treating or avoiding disease, notably those offered by modern molecular and cell biology.

These are not academic issues. Even if the NIH budget were infinite, the future pattern of medicine would still be determined by the present distribution among research fields of the limited stock of able people. But the budget, although increased by nearly \$1,000 million above the administration's request for the current year, is far from infinite. And resentment persists that, in the financial year to last October, there were fewer new research grants than in any year since 1977, and that the chance that a technically competent research proposal would be funded had fallen to less than a quarter. But the NIH budget is also constrained internally (by NIH's in-house commitments) and externally by the increasingly specific conditions attached by the US Congress to its allocations of funds, generous though they may be. This year, for example, NIH have been asked to increase the number of new research grants to 6,000.

There will be unwelcome consequences if it is left to the Congress to decide between these (and other) legitimately competing views of how NIH should spend their money. Last month, the Federation of American Societies of Experimental Biology went out of its way to reject the Institute of Medicine's plea on behalf of the infrastructure of biomedical research. Meanwhile, the American Society of Cell Biology, although keen that there should be more research grants, has been pressing that the promise of the new biology should be given more attention (see *Nature* 348, 270; 1990).

The obvious danger is that lobbying, successful or

otherwise, will tempt the Congress to determine not just the scale, but the details, of NIH spending. The growth of the NIH budget so far owes much to the touching faith of the Congress that more research means better health. Congressional committees have mostly allowed the research community to make its own judgements on the spending of public funds, confining their enquiries to administrative matters (such as the effectiveness of peer-review) and the good sense of major initiatives (such as the Human Genome Project). But the Congress could in principle take to offering opinions on the merits of certain kinds of research proposals. It has a constitutional right to a view on the infrastructure question, and on the potential benefits for health care of modern molecular and cell biology. But deciding these questions in isolation would be dangerous.

The best course would be that the long hiatus in the appointment of a director of NIH should be ended quickly, and that the new administration should undertake the long-overdue policy appraisal that would both carry weight with the research community and assure the Congress that the world's biggest fund for basic research is being spent wisely and effectively. In everybody's interests, it is important that two nettles should be firmly grasped. First, there is the running sore of what are called "indirect costs" — the overhead charges paid to institutions in receipt of research grants. The second nettle is the balance between in-house and extramural spending. To raise the question now is not to suggest that the present balance is wrong, but merely to remark that the question has not been asked, or answered, for a long time. □

Regional restraints

The ending of the Cold War seems to have revived interest in regional arms control arrangements.

THE governments of Argentina and Brazil have been blazing an interesting trail in the past few weeks. First, the newly elected president of Brazil ceremonially emptied the first shovel-full of rubble into a hole in the ground said to have been intended (by a secret agency of the previous government) for an underground nuclear explosion. Now Argentina and Brazil have jointly forsworn the development of nuclear weapons, but have also declared that



they will not sign the Nuclear Non-Proliferation Treaty (NPT) on the grounds that it discriminates in favour of established nuclear powers. Is nuclear proliferation going out of fashion?

Not necessarily. Indeed, manifestly not. Argentina and Brazil have been talking about their nuclear ambitions for nearly a quarter of a century. They have conspicuously been non-signatories of the NPT since its beginning in 1970. They are also signatories of the Tlateloco Treaty that would long since have made Latin America a nuclear-free zone, kept that way by mutual inspection, if only its members (which include France) had chosen to ratify it. Argentina and Brazil, ambivalent though they have been, have evidently come round to recognizing the benefits of avoiding the development of nuclear weapons, yet it is also plain that they have no intention of signing the NPT at this late stage. Most probably, the ceremonies of the past few weeks have more to do with growing confidence in elected civilian governments than with strategic interests which, for the time being, are regional.

Complaint

Yet the nuclear powers should pay more than their customary attention to the complaint that the NPT is discriminatory.

Technically, the complaint is true. The NPT requires that the civil nuclear facilities of only non-nuclear states should be inspected by the International Atomic Energy Agency. The stock explanation of the asymmetry is that the non-nuclear powers are offered in return both technical assistance in civil nuclear power and the promise of superpower agreements on strategic arms.

The trouble is that technical assistance has not materialized (and might not now be welcome) while the bilateral agreements between the United States and the Soviet Union, impressive in themselves, leave three acknowledged nuclear powers (Britain, China and France) and several putative nuclear powers on the loose. What can be done to take the edge off the discontent thereby engendered? Regional agreements are a valuable first step. Indeed, inspection by near-neighbours (who have the most to lose from inattention) may be more effective than inspection by an international authority. But such arrangements will command confidence only when they are validated internationally.

That point is underlined by the information, also made public last week, that governments such as that of the United States are paying attention to security arrangements and the regulation of weapons systems in the Middle East when (and if) the Gulf crisis ends. No doubt such a pact, which is apparently to be sweetened by proposals for economic development in the region, would either underwrite existing frontiers or specify the rules for changing them peacefully. There would also have to be analogues of the agreements on conventional arms now coming into force in Europe.

Whether the sovereign and often quarrelsome states of the Middle East would give each other mutually convin-

cing undertakings of this kind is, of course, a matter for conjecture.

Difficulty

The more serious difficulty is that Iraq, a member of the NPT, cultivates gossip about its interest in nuclear weapons. Israel, a non-member, has so successfully done the same for so much longer that its neighbours would be fools to behave as if Israel does not have a stockpile of nuclear weapons somewhere. But regional security in the Middle East would be a mirage without an understanding on more sophisticated destabilizing weapons such as nuclear bombs, not to mention chemical weapons (the poor man's interim and uncertain substitute for them). Making Israel toe that line will not be easy; asking somebody to give up something he does not acknowledge having is a delicate business. But Israel's compliance would probably be won more easily if responsibility for the verification of a nuclear-free Middle East were in the first place a regional responsibility. Naturally, international validation would be necessary if others were to sleep soundly at night.

So there seems to be in prospect a return to the 1950s fashion for regional security and arms control arrangements. It has been engendered by the ending of the Cold War. Although European confrontation was then the most likely cause of conflict in the bipolar world from which we may have emerged, the consequences would inevitably have been global. The alarming prospect of generally as well as mutually assured destruction, which may have kept the peace in Europe for 40 years, also helped to tamp down potential conflicts elsewhere. Iraq's adventure in Kuwait would have been an even greater risk if the classical superpowers were still locked in arm-wrestling.

Those changed circumstances bring dangers of their own. Although the United States and the Soviet Union remain united, as they were throughout the Cold War, in their distaste for the spread of nuclear weapons, their defence commitments to others are now less extensive and less zealous. So previously dependent states conclude that they must fend for themselves, ideally by cobbling together a bomb or two.

The best counter would be a demonstration that regional security arrangements can indeed be made to function. For Europe, the treaty on conventional weapons signed last month in Paris is an important step in that direction, while the 1988 treaty on intermediate-range missiles and that promised for next year on strategic missiles will take the edge off the nuclear problem.

But only partially. The interested parties, which means the European states and the classical superpowers, have yet to make a serious start on the control of battlefield nuclear weapons in Europe, let alone on regulating the strengths of the British and French strategic forces. The sooner they do so, the sooner people elsewhere will regard the trend towards regional security as the beneficial opportunities they are. □

German unity threat to space spending

- New government reviews space policy
- Physicists condemn spiralling costs

Munich

THE rumblings about Germany pulling out of the European manned space flight programme became louder last week, as politicians indicated that critical decisions on the space policy of the newly united country will be made in the next six weeks and the reunified German Physical Society (DPG) launched a stinging attack on the programme's scientific merit.

At stake is at least one of the three pillars of the European manned space flight programme, Columbus, which will form a module of the US space station Freedom. The Germans have a 38 per cent stake in Columbus. France has taken the lead in the other two projects: the space shuttle Hermes and the heavy payload rocket Ariane 5.

If the expected costs for the three projects rise more than 20 per cent from the original estimates, which they are quite likely to do, European Space Agency (ESA) member states have the option to withdraw. The 13 member states will meet to discuss the three projects by June 1991.

At the very least, Germany is planning to demand the 'stretching' of the Columbus programme over a longer period in order to spread the cost. The cost of the three projects, estimated in 1987 at DM21,000 million (\$14,190 million), has now risen to an estimated minimum of DM30,000 million.

"We have to ask ourselves whether it is good for Germany" to continue with the manned space flight programme, says parliamentary research committee member Heinz Seesing of the Christian Democratic Union (CDU), which with two smaller parties won a clear majority in elections on 2 December. Bearing in mind the uncertainty caused by the cost of German reunification, "we have to ask ourselves if we wouldn't be better off spending our money on microchips", adds Seesing. Nevertheless, he emphasizes that the final decision has yet to be made.

Seesing expects the position to be clarified during the coalition negotiations which are due to be completed by the end of January. The election "changed the face" of the parliament, he says. There are more than a hundred new members from eastern Germany who may not see the point of sending men into space while unemployment rockets at home.

Another parliamentarian, Jürgen Rüttgers, CDU space policy spokesman, says that the decision on Columbus will

strongly depend on the changes that NASA (US National Aeronautics and Space Administration) makes in the plans for space station Freedom following the critical report on NASA's activities by the Augustine Commission published last week (see *Nature* 348, 569, 1990). For example, if the power available for Columbus were to be reduced by half, as has been discussed, then it might be "less sensible" for Europe to build it. "Figuratively speaking", says Rüttgers, "if NASA lets us use the module for only half the day, then we have to ask if the whole project makes sense."

In the case of Hermes, Rüttgers says that Germany will wait for France to take the initiative in maintaining the current programme or reducing it to a technology project, focused perhaps on hypersonic transport instead of shuttling astronauts into space. "We will not pull out", he says. But of course if Columbus is reduced or eliminated, Hermes will lose its main *raison d'être*.

Smelling blood for the first time in its behind-the-scenes battle against manned space flight, the science community, led by the DPG, decided to make public its concern. It issued a damning broadside in the form of a memorandum condemning further German participation in the projects if the costs indeed rise by more than 20 per cent.

DPG urged politicians to stop arguing that science and industry will be the main beneficiaries of a decision to establish an independent manned space programme. A poll of DPG members, including those involved in microgravity research, which is often named as a potentially important user of the space station, revealed that the "overwhelming majority" opposed going further with the manned space programme, says Erhard Keppler, a coauthor of the memorandum and a physicist at the Max Planck Institute for Aeronomy.

As in the United States, researchers are mainly worried about money drying up for Earthbound research. They claim that the cost of space-based research is disproportionate to its benefits. Keppler says that Germany will have to pay as much as DM2,000 million a year just to keep Columbus in operation (the government estimates DM1,000 million), a huge amount compared with an annual 'basic research budget' Keppler estimates at DM16,000–17,000 million.

Rüttgers replies that such an

PARTICLE PHYSICS

Poland to join strike-disrupted CERN

London

POLAND will become the sixteenth full member of CERN, the European particle physics centre in Geneva, in July 1991 — the first of the newly democratic countries of Eastern Europe to join the organization.

Taking account of the country's severe economic difficulties, CERN's governing council decided last week that Poland should initially pay only SFr1 million (about £400,000) a year towards CERN's running costs. But the Polish contribution will increase to about 1.2 per cent of the total budget (SFr868 million in 1991) by 2000. Czechoslovakia, Hungary and Yugoslavia are also interested in joining CERN.

But CERN experiments may be disrupted by industrial action next year, after the council refused to agree salary increases for CERN staff beyond a 2 per cent increase in real terms in the coming year. The staff association is fighting for a 6.4 per cent increase by 1993, and has already introduced a work-to-rule (see *Nature* 348, 379; 22 November 1990), now suspended, and staged a number of two-hour strikes. Henry Atherton, vice-president of the staff association, says CERN staff are now considering what action to take during the next round of pay negotiations, which begin in the summer. Peter Aldhous

"unpolitical" argument "could only come from professors". There is no requirement for this money to come out of the Research Ministry budget, he says. Rüttgers believes that Research Minister Heinz Riesenhuber (CDU) will keep his promise not to let the space programme take up more than 22 per cent of the ministry's budget. At present, the figure stands at 20 per cent.

Keppler condemned the idea of 'stretching' the space projects over a longer time, because he says that it would make the "mid-1980s" technology, which he calls "the junk from the day before yesterday", even more obsolete by the time it arrived in space.

According to Rüttgers, the physicists are increasingly being joined by politicians in debating manned space flight in terms of its scientific merits instead of just talking about the desirability of "putting more Germans into space". He adds: "Manned space flight is not a dogma".

Rüttgers discounted the possibility that Germany would turn to the Soviet Union for more cooperation in space if the United States reduces its space programme. "The Soviets have a space station and a big rocket and they can put men in space", he says. "So what? We still have to ask ourselves why we want them there."

Steven Dickman

Rebuilding NASA's labs

Washington

OVER the past decade, while the National Aeronautics and Space Administration (NASA) was staggering under a series of debilitating technical and management failures, its research quality appears to have suffered as well. From the mid-1970s to the mid-1980s, overall NASA research citations dropped from above to below average in their fields, according to a new study by the Philadelphia-based Institute for Scientific Information (ISI).

Of NASA's eight largest laboratories, half saw a fall in citation levels from the 1970s to the 1980s. Some others remained solidly mediocre, consistently producing papers that earned fewer citations than other papers in the journals in which they were published. Overall, NASA research declined 10 per cent during the first half of the 1980s, according to the ISI study, which will be published in *Science Watch* later this month.

But among the sobering statistics, one NASA facility — the Jet Propulsion Laboratory (JPL) — showed a consistent improvement over the decade. That JPL is the only NASA laboratory to be run by a university, rather than the space agency itself, may suggest how NASA can best rebuild its research structure in line with the conclusions of the Augustine panel, commissioned by the White House, which released a preliminary report last week (see *Nature* 348, 569; 13 December 1990). Among its recommendations, the Augustine panel suggested that NASA begin the gradual conversion of some of its centres to become university-affiliated laboratories like JPL, as part of the process of refocusing the space agency's priorities back on science.

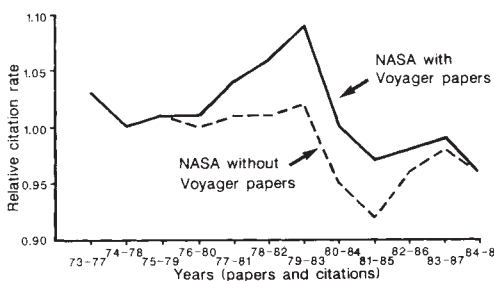
The problem with NASA's research, as the Augustine panel saw it, is that its researchers are civil servants, who have neither the responsibilities nor the incentives of university scientists. "The personnel policies embodied by the civil service system are, in the opinion of the Committee, hopelessly incompatible with the long-term maintenance of a leading-edge, aggressive, confident and able work force of technical specialists", the report concludes.

JPL researchers, on the other hand, work for the California Institute of Technology, not NASA directly. When the newly formed space agency adopted JPL in 1958, it agreed to pay CalTech a fee to run the laboratory. As a result, JPL's 7,000 researchers and technicians retain many of the employment perks of university researchers. Perhaps more importantly, they also know they can be dismissed if their performance is below par. At JPL there are no unions and few of the job protections that make dismissals so

difficult in the civil service.

While the rest of NASA was in the throes of its post-Apollo slump, research quality at JPL improved by more than 15 per cent, according to ISI analyst David Pendlebury.

Much of JPL's research success is due to its operation of the Voyager series of planetary probes, which surpassed all expectations in more than a decade of exploration. Similarly, some of the decline in research quality at other NASA laboratories is no doubt due to the shortage of



Impact of NASA articles with and without Voyager papers. The years indicate the year of publication and the following 4 years during which citations were counted. Citation per NASA paper relative to average citation per NASA journal paper.

good missions; the Challenger space shuttle accident and budgetary cutbacks conspired to keep NASA from launching a major mission for nearly a decade after the Voyager series. During that dry period, NASA laboratories such as the Johnson Space Center, which had scored impressive citation rates from its studies of the lunar samples returned by Apollo, floundered.

But in the view of the Augustine panel, management issues were at least partially responsible for JPL's success, as well as the sluggish performance elsewhere in NASA. The panel suggested first that the government reform its regulations to exempt researchers from the salary caps and other restrictions of the civil service. Should that prove difficult, NASA should try to put at least 10 per cent of its own employees under a specially tailored system to encourage scientific quality, the panel said.

Both of those suggestions have been raised before, but are generally considered to be politically unacceptable.

The third possibility — converting more NASA laboratories to fit the JPL mould — is more likely to get a sympathetic ear. But would more university-operated laboratories turn NASA's research decline around? Former JPL director Bruce Murray is sceptical. The laboratories that are best suited for university management are the ones in least need of improvement, he says. The Ames Research Center has ties to nearby Stanford University that could be ex-

panded and the Goddard Space Flight Center could be operated by the Johns Hopkins University, which already runs the Defense Department's Applied Physics Laboratory. But both Goddard and Ames have remained relatively steady in research quality in recent years. "You'd be fixing the labs that have the least need to be fixed", Murray says.

On the other hand, the weakest research centres on the citation criterion — Johnson and the Kennedy Space Flight Center, where NASA does much of its space medicine research — are large facilities where science takes a back seat to the design and construction of spacecraft. The probable incentive for a university or consortium of universities to take over such a centre would be profit, rather than prestige or research ties.

A more likely scenario, Murray says, would be for NASA to turn some of its laboratories over to private contractors, as the Department of Energy has with some of its national laboratories. That would permit industry-like management policies without requiring the unlikely reforms of the civil service that were recommended by the Augustine panel.

Even that change may prove difficult, however. Another concern expressed by the Augustine panel was that NASA headquarters appears to have lost control of its centres, allowing research overlap and a general blurring of focus. Contractor-run centres would probably be even stronger and more independent, notes John Pike, space policy analyst for the Federation of American Scientists. "NASA is a collection of spare parts flying in close formation, rather than a complete airplane", he says. "If you turn the field centres over to the contractors, that could just make the situation worse." He suggests increasing the number of political appointments at NASA headquarters as a means of giving the agency more clout — perhaps enough to rope in even contractor-run centres.

In the cold light of the statistics documenting a research decline, increasing congressional pressure for reform, and now the hard-hitting Augustine report, NASA would appear to be poised on the verge of a major restructuring programme.

But long-time observers warn that the most likely immediate action may be no substantial action at all. NASA has so far dismissed most of its failures as the result of congressional or White House meddling, or of past problems now fixed.

Accepting the need for fundamental change could take something as drastic as yet another disaster. "This is just a stage in a growing crisis, not the end", says one former NASA official.

Christopher Anderson

SPACE RESEARCH

Joy at last for ESA's science

Munich & London

AFTER years of argument and uncertainty, the European Space Agency (ESA)'s council last week agreed to increase the agency's budget for space science in 1993 and 1994, dispelling fears that important projects would be cut because of a lack of funds. The agreement comes as a welcome surprise for European space scientists, because many national delegates, particularly the British, were reluctant to agree increases to the science budget unless convinced that ESA was enacting measures to reduce unnecessary costs (see *Nature* 348, 474; 6 December 1990).

The 13 member states agreed to increase spending on science by 5 per cent a year in real terms in both 1993 and 1994, reaching a target of 260 million accounting units (MAU) a year (about £180 million) at today's prices. A further 8 MAU has been found for the Hipparcos star-mapping project in 1991. The operation of Hipparcos is costing much more than expected because the satellite has to communicate with three ground stations, rather than one, after failing to reach a geostationary orbit after launch.

The key to the new budget agreement was a resolution requiring Jean-Marie Luton, director-general of ESA, to reduce the overheads charged to the science programme for the use of ESA facilities. This should release an extra 15 MAU a year for science from 1992. Luton also has to streamline the programme's management, with the aim of saving a further 10 MAU a year. British National Space Cen-

MANNED SPACEFLIGHT

tre officials are delighted with the assurances to increase cost efficiency: they had drafted the resolution before travelling to Paris for the council meeting, but were unsure if other ESA states would support it.

The cost-cutting measures agreed were both recommendations of an independent review commission led by Klaus Pinkau of the Max Planck Institute for Plasma Physics near Munich. But member states balked at endorsing the most politically sensitive of the Pinkau recommendations — the relaxation of the strict rule that awards equipment manufacturing contracts to ESA member states in proportion to their financial contributions. This was rejected by the less industrially developed ESA nations, which joined the agency to obtain a guaranteed supply of manufacturing contracts for their fledgling space technology industries.

Roger Bonnet, ESA's director of space science, says the meeting was "a tremendous success". He is particularly pleased with the British endorsement of the increased budget — the long-running crisis surrounding ESA's science budget was precipitated by Britain's refusal in 1987 to fall in line with the other 12 member states over budget increases.

The ESA space science programme includes both joint European-US projects such as the ESA Huygens probe to Titan on the US Cassini Saturn mission planned for 1997 and solely European missions, including the Infrared Space Observatory, due for launch in 1993.

Steven Dickman & Peter Aldhous

Juno mission rides again

London

THE first-ever British astronaut will fly to the Soviet Mir space station in May 1991, now that a package has been put together to rescue the Juno mission, which collapsed during the summer after failing to attract commercial sponsorship (see *Nature* 346, 781; 30 August 1990). But the future for the programme of microgravity experiments which was to have accompanied the astronaut is still in question.

The mission's original financial backer, the London-based Moscow Narodny Bank, is paying an undisclosed sum of money to NPO Energiya, which builds launch vehicles for the Soviet space programme, to ensure a flight for one of the two British astronauts still in training near Moscow. Tim Mace, a major in the Army Air Corps, and Helen Sharman, a food technologist, must wait until next month before the final astronaut selection is made.

The bank says that Juno will have a science programme, but this may now consist of Soviet rather than British experiments. Heinz Wolff, from Brunel University, science director for the original mission, says he needs £250,000 to run eight of the 18 experiments that were scheduled to fly. But Wolff is having difficulty attracting sponsors, because the bank has provided little information on the exposure that sponsors will receive.

Wolff says that it is important that Juno should be seen as a "serious British scientific mission", after the "negative publicity" attracted by the recent flight to Mir by a Japanese astronaut. Toyohiro Akiyama, a journalist with the Tokyo Broadcasting System television company, spent much of his time in space informing the Japanese public about his space sickness and Mir's poor toilet facilities (see *Nature* 348, 575; 13 December 1990).

Peter Aldhous

ECOLOGY

All under control

London

BRITISH ecology will celebrate the New Year with the opening on 9 January of a unique ecology research facility at the Centre for Population Biology. The new £1-million facility, the Ecotron, will make it possible to model complete ecosystems in precisely controlled environments.

At the core of the Ecotron are 16 walk-in chambers with computer-controlled climates. These chambers will be used to establish self-sustaining communities of animals and plants, allowing researchers



to study the assembly of ecosystems, the make-up and stability of food webs, and the ways in which populations interact at different spatial scales.

Although controlled environment chambers are widely used in ecological research to study specific interactions, the Ecotron is the first facility to allow researchers to control model ecosystems precisely. In the longer term, scientists want to use the Ecotron to study the ecological effects of global warming or climate change and pollution, and possibly also the interplay between genetics and population dynamics.

The Ecotron is the centrepiece of the Centre for Population Biology at Silwood Park, near Ascot. The centre, which is part of Imperial College, was set up in April 1989.

Rory Howlett

EDUCATION

Reading the stars

New Delhi

INDIA'S Department of Space (DOS) has drawn up an ambitious plan to use space technology to tackle the country's illiteracy problem. It aims to launch a pair of satellites dedicated to beaming classroom education to every rural household. The programme, known as Gramsat (*gram* is Hindi for village), is aimed primarily at the inhabitants of India's 560,000 villages, 70 per cent of whom are illiterate. Gramsat will be unique in providing education in the mother tongue.

The two Gramsat satellites will cost \$150 million, according to Dr U. R. Rao, who heads DOS. He expects the first satellite to be launched in 1995. Formal government approval is awaited.

K. S. Jayaraman

Toshiba sets up UK chip lab

London & Tokyo

TOSHIBA, the Japanese electronics company, is opening a semiconductor research centre in Cambridge, England, in January 1991, its first European basic research laboratory.

The centre is one of a series of British-based research laboratories being set up by Japanese electronics companies, filling a vacuum in British semiconductor research left after recent cutbacks by British and European companies (see *Nature* 347, 415; 4 October 1990). Hitachi already has a seven-strong research group working within the University of Cambridge's Cavendish Laboratory, and Sharp, which recently opened a 12-man laboratory in Abingdon near Oxford, is planning to expand into a large centre in the Oxford Science Park in 1992.

The Toshiba Cambridge Research Centre has an initial research and development budget of £640,000 a year, and will concentrate on quantum effect physics — which may underpin the next generation of miniaturized electronic devices. Michael Pepper, professor of physics at the Cavendish Laboratory, will be the centre's director, and says the ten researchers he is recruiting will collaborate closely with the university. Sei-ichi Takayanagi, senior vice president of Toshiba, says the centre's staff will be allowed to publish their results freely, and any patentable technology will belong to the centre.

Japanese companies are keen to invest in research in Britain to marry British expertise in basic research with the Japanese flair for technological development. A longer-term aim is to strengthen the vertical integration of their operations in Europe from basic research to production. Toshiba also plans to establish product development laboratories at some European production facilities.

Another motivation is Toshiba's desire to collaborate in European Communities (EC) research projects. Takeo Fujii, European general manager for Toshiba, says that the company has approached European Commission officials, but has not yet discussed any "concrete programmes".

As a subsidiary of Toshiba registered in a European country, the new centre would be eligible to participate in EC research projects. But the problem will be finding European companies willing to collaborate with a Japanese-owned company. Some European computer companies are now questioning their continued collaboration with the British company ICL in a number of ESPRIT information technology projects, after the sale of a majority holding in ICL to the Japanese company Fujitsu. Toshiba officials are aware of the potential problems, and stress that their centre will be devoted entirely to basic research, which should be less commercially sensitive.

Peter Aldhous & David Swinbanks

OFFSHORE AIRPORT

That sinking feeling . . .

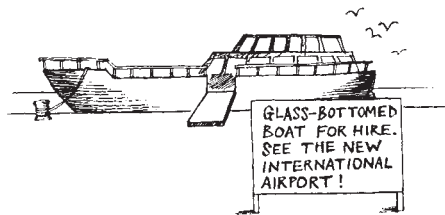
Tokyo

A RED-faced Japanese official in charge of plans to build the world's largest man-made island in the Seto Inland Sea off Osaka had to admit to the foreign press on Monday that the opening of a new international airport on the island will be delayed because the island is sinking into underlying mud much faster and further

embarrassing because foreign construction companies were shut out of the lucrative reclamation phase of the \$11,000-million airport project because of (among other reasons) their "inexperience" in dealing with the soft marine sediments around the coast of Japan.

KIAC took extraordinary measures to strengthen the sea bed at the construction site in 18–20 metres of water 5 km off the coast when reclamation work began in 1987. But the company failed to predict correctly the amount of compaction in the much deeper underlying Pleistocene layers of sediment at 20–400 metres below the sea floor as the newly emerging 511-hectare artificial island presses down with a force of 40 tons per square metre.

Before construction, KIAC estimated that subsidence in the Pleistocene layer would be only 1–2 metres but actual subsidence will be more like 5–6 metres according to measurements of settlement of the island. As a result, an extra 3.5 metres of fill will have to be added to the island at a cost of about ¥20,000 million (\$150 mil-



than had been predicted (see *Nature* 347, 7; 1990).

Yoshio Takeuchi, president of the Kansai International Airport Co. (KIAC) announced with "great regret" that the scheduled opening of the airport in March 1993 will be postponed by "about 15 months". The delay is particularly

SMALLPOX VIRUS

Lab stocks to go

London

THE last remaining stocks of the smallpox virus should be destroyed by the end of 1993, after US and Soviet laboratories have finished sequencing the smallpox viral genome. Frozen viruses are now kept only at the Centers for Disease Control in Atlanta and the Research Institute for Viral Preparations in Moscow. The thaw in superpower relations is a factor in the decision to destroy the stocks — both countries had feared that the virus could be used in biological warfare.

Smallpox was eradicated outside the laboratory in 1977, after a decade-long campaign by the World Health Organisation. A single case occurred in 1978, through a laboratory accident in Birmingham, in the British Midlands.

Peter Aldhous

BSE

Maternal transmission in antelope

London

AGRICULTURE ministry pathologists have confirmed that an 18-month-old kudu from London Zoo, never fed the animal feed thought to contain the infective agent of bovine spongiform encephalopathy (BSE), died of a BSE-like disease. The only source for infection seems to be the antelope's mother, which died of a spongiform encephalopathy in 1989. This is the first time a BSE-like disease has been shown to be transmitted from mother to offspring, except from in scrapie-infected sheep, where maternal transmission is common (see *Nature* 348, 380; 29 November 1990).

The finding increases speculation that maternal transmission may also occur in BSE-infected cattle. But an agriculture ministry spokesman says that farmers will still not be advised to avoid breeding from the offspring of BSE cases. Even if maternal transmission of BSE is possible in cattle, it may not prolong the epidemic significantly if it is a reasonably rare occurrence.

Peter Aldhous

lion) to keep the airport above sea level.

Furthermore, the foundations of the Italian-designed airport terminal building will have to be reinforced with iron ore and the two wings of the terminal will be mounted on hydraulic jacks to compensate for differential subsidence of the wings after the airport opens.

But by far the biggest cost of the delay will be interest payments on the huge loans taken out by KIAC to finance construction. All in all, the delay is expected to cost more than ¥100,000 million (\$750 million) . . . plus a certain loss of face.

David Swinbanks

RESEARCH FUNDS

Hughes reaches out

Washington

AFTER of years of appeals from abroad, the largest private source of research funds in the United States is planning to go international.

The wealthy Howard Hughes Medical Institute (HHMI) has agreed to begin funding research in Canada and Mexico, and hopes to expand across the Atlantic in 1991, officials said last week.

An HHMI external advisory panel will decide the exact amount of the funding and number of grant recipients in Canada and Mexico at a meeting next month, and a formal announcement will be made in April, according to Purnell Choppin, president of HHMI.

The new programme is expected to be the first stage of an international expansion for the foundation, but HHMI officials say that it is too early to pinpoint where the institute's largesse will land next.

FETAL CELL TRANSPLANTS

French fetal cell transplant operations

Paris

A FRENCH national ethics committee for life sciences and health has overturned its ban on the use of fetal brain tissue grafts for medical treatments.

This means that a team of doctors at the Henri-Mondor hospital in Créteil, south of Paris, will now be able to carry out pilot operations to treat patients suffering from Parkinson's disease.

This kind of treatment is now authorized in some other European countries including Britain, Spain and Sweden, but is allowed in the United States, where there is a powerful anti-abortion lobby, only so long as federal funds are not used.

In October last year, the ethics committee ruled that more research was needed before fetal tissue could be used for this kind of operation. At that time, fetal dopamine-producing cells were used to enhance the effects of adrenal tissue extracted from the patient and reinjected into the area of the brain where dopamine is produced.

This is a very dangerous procedure. But, says Marc Peschanski, a researcher at the Henri-Mondor hospital and co-signatory of a recent appeal to the ethics committee, new research in Sweden has made the operation safer.

According to Peschanski, the ethics committee's 1989 ruling was unsolicited. "We were not even consulted", he says. But when his research team protested, the committee agreed to review its position. Now it is Peschanski's proposed protocol that has led to the ethics committee's about-turn.

Peter Coles

Joseph Perpich, HHMI's vice president for grants, said that the programme was in part a reflection of a "large number of entreaties from abroad", especially the United Kingdom. But a decision on where and when to expand the programme beyond North America will probably not be made until late in 1991, he said.

"We decided to start this on a pilot experimental basis and the logical first choice was our immediate neighbours", Choppin said. Future expansion may be in both the developed and developing world — "anywhere where the best science is going on". Rather than opening the competition to all, HHMI plans to target specific geographic areas. "We're not going to deal with the whole world at the same time", he said.

HHMI spends some \$300 million (4.5–5 per cent) of its \$6.1 million total worth each year on basic biomedical research.

HIV INFECTION

UK haemophiliacs win compensation

London

IN A sharp reversal of government policy, John Major, the UK Prime Minister, last week announced that haemophiliacs infected with the AIDS virus through imported blood-clotting factor VIII supplied by the National Health Service will receive a further £42 million.

David Watters, general secretary of the Haemophilia Society, welcomed the government's implicit admission of legal responsibility (previous payments of £34 million had been made on an *ex gratia* basis), but was "very disappointed" that the settlement fell short of the £100 million the society was campaigning for.

Nevertheless, most haemophiliacs and more than 200 dependents fighting the government for compensation are now expected to accept the settlement, averaging £35,000 each, and drop their legal action.

About 1,200 British haemophiliacs are now infected with HIV (human immunodeficiency virus), and over 150 have died from AIDS.

The government had previously maintained that scientific knowledge in the early 1980s, when the haemophiliacs became infected, was insufficient for it to be held responsible.

A number of HIV-infected haemophiliacs in the United States have filed negligence suits against individual pharmaceutical companies. But these are unlikely to be successful, as most haemophiliacs have used clotting factor produced by a number of companies (see *Nature* 347, 319; 27 September 1990). **Peter Aldhous**

To retain its tax-free status, HHMI distributes most of that in the United States to its special HHMI investigators, mostly university scientists who officially become HHMI employees. But some \$50 million is also spent annually on grants to other researchers, and all over-seas research support will probably come from that pot. First-year funding to researchers in Canada and Mexico has not yet been decided, but is likely to be in the range of "a few million dollars", Choppin said. The bulk of HHMI's expenditures, however, will probably always be in the United States, he added.

Potential recipients in Canada and Mexico have already been contacted. Typically, HHMI grants are in \$50,000–\$100,000 range.

Choppin said that HHMI is not at present taking applications for other international grants, beyond those for existing programmes to support foreign students in US universities and to send US students abroad.

Christopher Anderson

CANCER RESEARCH

ICRF in row over 'exaggerated' claim

London

AN Imperial Cancer Research Fund (ICRF) fund-raising advertisement carried by several British national newspapers "could be seen as claiming an exaggerated pioneering role" in the development of a breast cancer therapy, says the UK's Advertising Standards Authority (ASA). The ICRF, the largest UK funding body for cancer research, which relies on charitable donations, has rejected the criticism.

A number of medical scientists questioned the claim that an ICRF group at Guy's Hospital, London, pioneered conservation therapy — where iridium implants are used as an alternative to the psychologically distressing treatment of mastectomy.

The technique was first developed by the surgeon Sir Geoffrey Keynes, working in a private London practice in the 1920s, the complainants argued. The ICRF has no plans to re-use the advertisement, but stands by its content. Keynes's early work was inconclusive, the ICRF says, and the Guy's team played a key role in the largest clinical trial of conservation therapy.

The ASA is a voluntary regulating body set up by the British advertising industry, mostly dealing with objections to misleading claims made for commercial products. When complaints are upheld by independent experts, the authority asks for advertisements to be withdrawn. The ASA took a middle course in the case of the ICRF advertisement, delivering a simple admonishment rather than formally upholding the complaint. **Peter Aldhous**

Targets work — up to a point

Washington

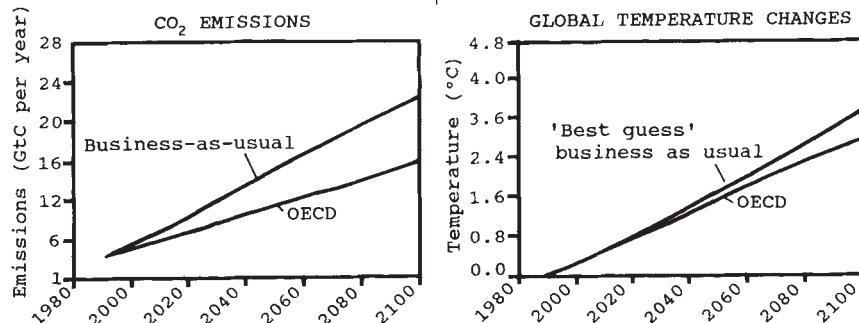
THE planned limits on greenhouse gases could cut the predicted global temperature rise by about half a degree by the year 2100, according to a study commissioned by the US newsletter *Global Environmental Change*. But the total temperature rise during this period could be as much as 4 °C, so the measures may not be enough significantly to slow global warming, the study by the Climatic Research Unit at the University of East Anglia (UEA), United Kingdom says.

By the end of this year, 22 industrialized nations — including almost all of the Organization for Economic Cooperation and Development (OECD) — will have adopted formal greenhouse gas stabilization or reduction targets. Assuming that all the countries stick to their goals, overall OECD greenhouse emissions

figures. But because the United States represents such a large fraction of OECD emissions, that increase is likely to increase total OECD carbon dioxide output by some 7 per cent over the same period, the study finds.

Even so, the predicted global output of greenhouse gases will fall over the period, reflecting the importance of chlorofluorocarbons (CFCs) in the equation. CFC limits imposed by the Montreal Protocol are the single largest factor in the projected reduction of greenhouse gases. Atmospheric concentrations of greenhouse gases would continue to rise, however, as the rate of new emissions still exceeds natural dispersion.

The researchers at UEA who entered the projected emission figures into their 'STUGE' computer model, project that the OECD reductions would result in



From the UEA study for the years 1980–2100. 'Business as usual' includes the revised Montreal Protocol cuts. OECD denotes effect of OECD greenhouse targets.

should drop 22 per cent by 2005, as measured by the World Resource Institute's greenhouse index, which weights various gases by their carbon equivalents (see *Nature* 347, 705; 25 October 1990).

Of the OECD countries whose carbon dioxide output is large enough significantly to affect global trends, only the United States is expected to increase its carbon dioxide emissions in the next 15 years — by some 19 per cent over 1987

0.45 °C less global warming, 7 cm lower sea rise, and a delay of eight years in the expected doubling of atmospheric carbon dioxide concentrations from a "business-as-usual" scenario in which no reductions are made.

But the report warns that atmospheric predictions are notoriously unreliable, and that these projections should be taken with a "healthy degree of scepticism".

Christopher Anderson

Indian team faces cold weather test

New Delhi

INDIA has sent its first research team to the Arctic on a mission to find out how people from warm climates acclimatize to extreme cold.

The expedition is organized by the Defence Research Development Organisation (DRDO) in collaboration with the Soviet Union's Arctic laboratory at Kirovsk. The team consists of ten soldiers from the Indian army and seven medical specialists from the Defence Institute of Physiology and Allied Sciences (DIPAS) in New Delhi, one of DRDO's 40 research institutes.

According to Dr J. Sengupta, director of

DIPAS, the expedition is the culmination of two years of negotiations with the Soviet Union. Eight Soviet scientists will take part in the experiments, which will compare data from the Indian subjects with those of ten Soviet volunteers to see if there are any differences in how the two groups respond to Arctic temperatures.

The cold acclimatization study could have been carried out in the Antarctic, where India has had a permanent research station since 1981, but Sengupta said this was not done for one simple reason. "Our Antarctic station is centrally heated, whereas the Soviet Arctic station is not."

K. S. Jayaraman

The gang that couldn't shoot straight

Washington

IF you had wanted to break into one of the most sensitive US weapons research facilities, April 1989 would have been a perfect time. For that month, and for a few weeks on either side, the US Department of Energy (DoE) was guarding its classified Los Alamos National Laboratory with a security force that was a little soft on some of the basics — shooting, handcuffing and stopping intruders. Many of the guards, who were replacements brought in during a ten-week strike by the usual security force, had not been trained to use their weapons, and none were issued with batons because they were not qualified to use them. A congressional oversight committee notified DoE that it had received allegations of "many instances of firearms 'horseplay' by the replacement force". During a simulated entry attempt, the guards failed to find drug equipment.

But a subsequent investigation by the General Accounting Office (GAO), Congress's investigative arm, revealed that the temporary guards were hardly worse than the officers they replaced. In fact, as a surprise inspection earlier this year revealed, 78 per cent of the regular force "lacked one or more of the skills needed to arrest, apprehend, communicate, and survive in an adversarial situation; protect laboratory resources or staff; or defend themselves", says a report published last week.

During a series of unannounced simulated break-ins, many of the current Los Alamos guards "left their cover and walked up to the potential adversary to ask what they were doing. As a result, in many instances the adversary took a visible weapon, 'killed' the [guard], and left with the classified documents of government property", GAO reported. In total, 24 of the guards were "killed" during the testing.

DoE, according to the report, "recognizes that these problems exist not only at Los Alamos, but throughout the nuclear weapons complex". As a result, DoE has asked its central training academy to reexamine the training given to its security forces, and to develop a standard course for security guards. And DoE may consider trying some more unannounced tests. Although DoE policies allow for such tests, it had not conducted them in the past because, as DoE officials explained to the GAO, "they raise safety concerns, are difficult to plan, disrupt the work force, and create stress for all participants".

Christopher Anderson

A genuine global collaboration

SIR—In his recent Commentary article entitled "A genuine global partnership?" (*Nature* 346, 692; 1990), S. S. Yamamoto expresses concern about the proposed Japanese participation in the Superconducting Super Collider (SSC) project. He refers to 91 Japanese physicists who "have agreed to participate in the SSC project", but suggests that they "will be paying an entrance fee to join the project without being able to make significant scientific contributions".

The 91 physicists to whom Yamamoto refers have joined the Solenoidal Detector Collaboration (SDC) which is preparing the design of a major detector for the SSC. As the spokesman for that collaboration, I would like to comment on the role being played by its Japanese members and correct some possible misunderstandings.

The SDC, formed about a year ago, brought together four separate groups of physicists working independently on similar detector concepts. Three of those groups were in the United States and the fourth in Japan. Japanese high-energy physicists had held a series of workshops, and their design efforts were the most advanced of any of the four groups. From the beginning, it was clear that, even more than financial capital, intellectual capital was needed, and Japanese intellectual capital has been crucial to our collaborative efforts. Indeed, the Japanese collaborations are making major technical and scientific contributions to the detector design, as well as playing an important role in the overall leadership.

Yamamoto states that "although the United States invites foreign participation where money and manpower are concerned, to the best of my knowledge they do not invite participation on an equal basis in scientific, technical and administrative matters". I cannot speak for the United States, but I can speak for the collaboration with which the 91 Japanese physicists are associated. The management of our collaboration consists of a spokesman and three deputy spokespersons (from the United States, from Europe and from Japan). Our governing board has 17 individuals, ten from the United States, two from Europe and five from Japan. We have numerous technical committees, each with several co-chairpersons — in all cases but one there is a Japanese co-chairperson. The oral presentation, made to the SSC Laboratory Program Advisory Committee on behalf of our detector concept, was divided between two people, the SDC Japanese deputy spokesperson and myself. To put all of this in perspective, our 91 Japanese collaborators represent about 20 per cent of the present total membership of the SDC, although they have made a substan-

tially larger fraction of the contributions (scientific and technical) to the work.

One major obstacle comes from the geographical dispersal of the collaborators, and the substantial travel costs in both time and money. But we can communicate almost instantaneously by electronic mail, and we hope that, in the near future, video-conference technology will permit frequent meetings between collaborators all over the globe.

On financial support, we are hoping to build an ambitious detector, and the costs will be high. This collaboration, if it is to fulfil its aspirations, needs financial support from Japan and from other non-US collaborators, and of course, it expects support from the US government. In large part, the Japanese contribution to the detector is likely to involve the design and fabrication in Japan of major pieces. To Yamamoto's advice that "the US government and high-energy physics community should insist that the Japanese contribution is scientific as well as financial", I can only say that this insistence is not necessary. Our Japanese colleagues, with many distinguished scientists among them, would not have it any other way.

As a citizen of the international high-energy physics community, I want to express my full agreement with Yamamoto that the continued development of future high-energy accelerator projects in Japan is indeed very important. At the same time, about a third of the Japanese experimental high-energy physics community is sufficiently excited by the scientific opportunities offered by the SSC to have invested a large amount of time and effort in beginning the preparation of SSC experiments. I share that excitement and I hope that with the support of the Japanese scientific community the Japanese government will find it possible, and indeed desirable, to provide the resources needed to bring these outstanding efforts to fruition.

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Group theory

SIR—British science and technology have until recently had an enviable record of innovation. We think this is due to the fact that they have been run on well tried and, until recently, trusted principles of 'backing the man'. Although this principle may have been elitist, it meant that science and technology were done by the individuals who knew their subject and were left to get on with the job.

The results speak for themselves. During a period of about 300 years, British

scientific institutions and in this century, the research councils have operated on the basis of "powerful informality", as one US philosopher put it, by backing individual thinkers whose work made significant contributions to science and consequently, to society as a whole. This approach paid for itself many times over and gave our system of science funding a world reputation second to none.

It is with some consternation, therefore, that we observe how deeply the recent politico-economic panic has affected the research councils and university funding bodies. First, there has been a change from 'backing the man' to backing the group (the takeover fallacy). It needs real knowledge to judge new ideas but anyone can count heads. Second, the Science and Engineering Research Council (SERC) is now asking for "estimates of proposed cash flow" as if the scientist were proposing to open a fast-food franchise and count the number of hamburgers crossing his counter. It seems that the council is being taken over by amateur accountants with attitudes of starry-eyed simplicity.

But the 'bottom line' is surely in the latest SERC guidelines for research applicants, where we are asked to "assume that the reader (of the grant application) knows very little about the subject"! What kind of refereeing is this? *Quis custodiet ipsos custodes?*

F. BROWN

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Women at work

SIR—At a recent primatology conference, organized at the University of California, Santa Cruz, only women were invited or allowed to attend. This highlights an important, but avoidable, problem in all scientific work: the experimenter effect or experimenter bias.

This occurs when an experimental outcome is not the result of the manipulation of independent variables, but rather results from some (typically) unintentional act or actions of the experimenter or experimenters. Scientific conferences should also be organized so as to ensure the accurate and reliable gathering, analysis and distribution of information. To conduct a scientific conference in 1990 from which male participants are excluded seems to imply that the results obtained, like an experiment confounded by experimenter bias, should be viewed with caution.

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Germany in perspective

SIR—The discussion following the remarks of Mr Nicholas Ridley on the supposed national characteristics of Germans (*Nature* 347, 510; 1990) is partly amusing but mostly disturbing. I believe that scientists should be discussing these matters, and perhaps should have done so more readily in the past. May I put the views of a physician-scientist of the post-war generation of Germany?

Anybody now articulating ideas of "national or racial characteristics" has either never been exposed to a foreign culture and country for any length of time, or has consciously or otherwise incorporated some of the ideas of Nazi ideology into his or her thinking. It is the very simplicity of these ideas that makes them so attractive and dangerous.

Ridley is merely one of a large number of people worldwide who, almost undisputed, hold that Germans are belligerent people forever eager to dominate their neighbours and the rest of the world, so that a strong and unified Germany is a potential threat to peace and security. Despite the destruction and suffering inflicted upon other people by Germans in this century, a sober view of history does not justify this conclusion.

In the seventeenth and eighteenth centuries, what is now Germany was a permanent battlefield upon which outside powers fought over their conflicting interests. As a result, many Germans settled peacefully, usually by invitation, in the Balkans and in Russia, where they maintained their culture and identity and were respected for their skills and hard work.

Sovereigns of the German states meanwhile sought to maintain a delicate balance of power among themselves. The contrast between the Germans in the seventeenth and eighteenth centuries and the Portuguese, Spanish, British, Dutch and French, who subdued and exploited other peoples, countries and continents to build colonial empires, is striking.

So vulnerable and comparatively peaceful were the German states that, early in the nineteenth century, the French armies of Napoleon swept across central Europe and overran even allegedly "militaristic" but hopelessly inferior Prussia. Only such formidable superpowers as Russia, Great Britain and Habsburg-Austria rescued the German states. The efficacy of the French armies (a "national characteristic?") made a lasting impression on sovereigns across Europe; a reorganization and build-up of armies began, particularly in Prussia. It is not by chance that most of the military terms in German derive directly from the French.

After the failure of the liberal democratic revolution in 1848 and with the faltering of the federation of non-Prussian

states (Deutscher Bund) under Austria's patrimony, Bismarck was able to unite the German states under the hegemony of Prussia. The wars fought to that end cost a tiny fraction of the lives and resources lost in the American Civil War; they also ended in honourable bilateral agreements and not in humiliation or annihilation of the adversary.

The new united Germany, under the guidance of prudent politicians, made central Europe a prosperous and remarkably stable region, so stable in fact that it also stabilized the neighbouring Russian and Austrian empires; both collapsed immediately with the fall of Germany and without it would probably have done so much sooner.

Whatever one may think of the follies of Kaiser Wilhelm II, his colonial adventures (who said "the Germans to the front!" anyway?) and his attempts at naval dominance which made Great Britain an ally of France, in 1914 Germany had no reason to go to war at all and was no more eager to do so than all the others involved, but found itself inextricably tied to the sinking ship of Habsburg-Austria by an intricate system of treaties and alliances.

In 1918, all the countries involved had suffered equally, but there was one important difference. While France and Britain imposed taxes to finance the war, thereby spreading the burden evenly, Germany's war effort was financed mainly by voluntary contributions as war-bonds; 1918 saw large segments of German society, especially the middle class, not only decimated by huge losses of men, but also deprived of their savings, humiliated and disappointed. Other sections of society had contributed very little and still others had made huge profits during the war.

A humiliated Germany in socioeconomic upheaval was not only a hotbed for the radical political movements that soon emerged but also an obvious threat to stability in Europe. The inability of our parents' generation, inside and outside Germany, to control Hitler and his followers and the consequences thereof is a fact of history but even there it is difficult to conclude that it was all due to "national characteristics". That, of the European dictators (who included Franco and Mussolini), Hitler and Stalin would finally engage in a deadly struggle reflects both the economic and geographical conditions of *realpolitik* in Europe as well as the innate characteristics of dictators and their relationship with each other.

The often-heard statement that the partition of Germany and the resulting cold war brought "peace and stability" to Europe can at best be called cynical. Apart from the suffering of people under Communist rule, the world was repeatedly

on the brink of nuclear war, the arms race brought two superpowers to their knees and the consequences, especially of nuclear armament, are only slowly becoming apparent.

If there is one traditional "value" that has got Germany and Germans into trouble, it may be our striving for perfection and overdoing things. I think we are learning that such extremism, while beneficial in science and industry, may well be harmful when applied to human affairs at any level.

The prospect for Europe today seems brighter than ever, but the formidable obstacles ahead will not be overcome nor the substantial problems solved by unearthing petrified prejudices and fossilized philosophies on "national or racial characteristics".

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Wittgenstein

SIR—I cannot agree with Marshall's opinion (*Nature* 347, 435; 1990) that Wittgenstein was only "a minor Austrian aphorist". I presume that, as a scientist, Marshall appreciates only a "positive" thought that can quickly lead to an advance in knowledge. Wittgenstein's thought, however, seems to lead nowhere. The philosopher was well aware of this: "I am not interested in erecting a building, but in seeing clearly before me the foundations of all possible buildings. My aim is different from that of the scientists and the movement of my thought is also different." (*Vermischte Bemerkungen*, Suhrkamp 1972.)

This way of looking at the world decreases the immediate heuristic value of Wittgenstein's work but, in my opinion, it by no means detracts from his philosophical merits. Human culture is made not only of scientific advances but also of seemingly insoluble problems deeply rooted in the structure of our thought and language.

The great merit of Wittgenstein consisted in showing us that these problems exist and that we really do not know what we are talking about, although, of course, we have to go on talking. Such language problems are put aside by scientists (otherwise there would be no progress), but their disturbing presence emerges again and again in science as well as in ordinary life. Perhaps the only way of solving them would be to communicate only by means of a mathematical language, a rather impractical task, I fear.

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The insignificance of personality testing

Steve Blinkhorn and Charles Johnson

The business world now sets great store by personality tests when assessing job applicants. But the evidence for their predictive value is frequently overstated and wrongly assessed.

PERSONALITY testing has never been uncontroversial in psychology. Many academic psychologists would be surprised to find that in the business world personality testing in recruitment and selection is considered to be the pith and essence of psychology. But in recent years there has been a dramatic growth in the use of personality tests for these and related purposes. About 50 per cent of companies in the United Kingdom claim to make use of personality tests at some point in their selection or assessment processes. Some of these tests have claims to origins in psychological theory; others make technical, methodological or empirical claims; still others rejoice in being unencumbered by any such distracting irrelevances. But even where the proposed basis for their use is demonstrated empirical predictive utility, we have severe doubts as to the accuracy of the claims.

The normal basis for a discussion of the use of psychological tests is statistical findings, usually expressed as correlation coefficients. But many proponents of personality testing adopt an approach to correlation that would have left its inventor Carl Pearson gasping and which beggars the contribution of R. A. Fisher to significance testing. But few of the punters have the kind of background in statistics that enable them to evaluate claims, and the numbers can be made to look truly impressive.

Caricature

Personality tests are scarcely new. Their origins in the first half of this century can be caricatured as a response to the apparent success of intelligence testing. "We have the technology" came the cry, and elder statesmen, now revered, turned their skills, their factor analytic routines and their research grants, to investigating the structure of human personality through the medium of questionnaires. It is not all that they did by any means, but it is the part which has survived to fascinate and intrigue. And although the popular imagination may focus on Rorschach ink-blots and the like as the type for personality tests, the big money is with multiple-choice questionnaires.

Early systems for describing the structure of human personality showed a certain enthusiasm for scientific mystification. How do you rate on Rhythymia, Hypochondriasis and Affectia? More recently there has been a fashion for gritty

everyday language. Submissiveness, Independence and Data Rationality. But regardless of terminology, the evaluation of the validity of a test has been conducted in terms of correlation coefficients.

For many years we took the view that the relatively poor showing of personality tests as compared with tests of mental ability was down to the need for extensive technical improvement. Ability tests show a rather monotonous tendency to correlate around 0.30 with various job performance measures. Personality tests do distinctly worse. Reasonable suggestions were made that the problem lay in: details of construction leading to poorer psychometric properties; the clinical orientation of many of the items in tests; the scarcity of well-conducted validity studies; the use of discredited theoretical approaches to the structure of personality.

All of these observations may well have been accurate, but all have now been addressed, and word is abroad that the current generation shows distinctly better validity than what went before. We beg to differ. We have conducted an informal survey of research on the predictive validity of three of the personality tests most widely used for employee selection and assessment which have claims to a serious and respectable pedigree, and we find a disconcerting approach to the analysis and interpretation of statistical data.

It has to be said that in restricting ourselves to these three (the California Psychological Inventory, the 16 Personality Factor Questionnaire and the Occupational Personality Questionnaire) we were consciously choosing to operate at the top end of the market. We maintain a 'Black Museum' of tests guaranteed to terrify the methodologically squeamish. Most of these, because of the way in which they are constructed, yield scores which are mathematically interdependent, and so unsuitable for the usual forms of statistical analysis. But the three we chose to look at are serious measures, constructed by sober-minded teams aiming for quality rather than just a quick buck.

The general style of these tests is as follows. Some activity (for example, going to parties) or choice (for example, parties versus reading a book) is briefly described, and the candidate is asked to choose from a limited number of options which describes him or her best. Responses to perhaps 10 or 15 such items are scored according to predetermined rules

to make up one of the 'scale scores'. These scores can be presented in a graph (or 'profiled') or, increasingly, used to drive software to produce written reports.

The first thing that would strike a statistically competent but psychometrically unblooded observer is the sheer number of scores assigned on the basis of each of these tests — between 16 and 30. Linear algebra may make a great deal of sense as a way of capturing a handful of dimensions, but what precedents do we have for conceiving of a 30-dimensional space?

Big Bang

When, as is often the case, scores are expressed on 10-point scales, there is a simple calculation which dramatizes the point. Imagine a computer system printing individual reports based on personality profiles. It drives an 8-page-per-minute laser printer, and on average each report consists of eight pages. Being an exceptionally reliable piece of kit, this system needs no maintenance. We set out to print reports based on all possible profiles for 16 scales, and wisely do so at the time of the Big Bang. In round figures, as we enter the last decade of the 20th century, there are only 500,000,000,000,000 of the 10^{16} possible profiles left to print. The system in the next room, which is working on a 30-scale test, is barely into its first lap.

In truth it is difficult to know how one would justify the underlying mathematical model. We doubt that, even just splitting each scale at the mid-point to yield 2^{16} possible profiles, any existing database actually contains a representative of each. And in typical validation studies, we find samples of between 30 and 150 subjects.

But there is more. Only rarely are investigators satisfied with a single-criterion measure against which to validate their preferred test: five or six are not uncommon. The enterprise then becomes a fishing expedition. Take 30 test scores and half a dozen criterion measures on 50 or 100 subjects; calculate product-moment correlations between all scores and all criteria. Then look for your trusty table of critical values of the correlation coefficient and note and significance level of each. Report only those which are 'significant' at the 0.05 (or one-star), or 0.01 (two-star) or better level. Hey presto! Your test is now valid.

Do not on any account point out that you have calculated more correlations than there were subjects in your sample.

Never hypothesize in advance the magnitude or direction of the correlation, for that way your hypotheses may be shown to be wrong rather than merely not proven. Avoid mentioning how many 'nonsignificant' correlations were found.

We think that this kind of misuse of a hypothesis test is scandalous, and bamboozles an unsophisticated public with

appropriate null hypothesis for a list of many correlations is a run of zeroes, but of course it is not. As has often been noted, the distribution of sample product-moment correlations is complex, and depends on both sample size and the underlying correlation in the population. Assuming zero correlations in the population, however, we can derive an expected

TABLE 1 Expected and observed correlations between 30 test scales and overall job performance (sign and decimal point omitted)

Expected	39	33	29	26	24	22	21	19	18	17	16	15	14	13	12
Observed	32	32	32	28	26	23	22	20	18	18	17	16	16	15	15
Expected	11	10	09	08	08	07	06	05	05	04	03	02	02	01	00
Observed	13	13	13	11	10	09	09	08	07	07	06	03	03	02	02

pseudoscience. The distribution of correlation coefficients in multivariate designs is a rather intractable problem, but that is no excuse for pretending that it can be ignored, and that the repeated application of a test designed for a single correlation can simply be prolonged until a 'significant' result is obtained.

To get an empirical perspective on the issues we turned to published sources, and started counting stars. For instance, the publishers of the Occupational Personality Questionnaire have published a review of validity studies, of which 28 were germane to this issue. Variations in the way results are reported make it difficult to be precise, but on average in any given study about 6.5 per cent of correlations are marked as significant at the 5 per cent level or better. In other words, there appears on the surface to be some slight effect beyond what one would expect in a random system. But both test scales and criterion measures tend to be heavily correlated amongst themselves, which will tend to increase the incidence of significant correlations.

Elsewhere we have found 11 published studies concerning the California Psychological Inventory (CPI) and four concerning the 16 Personality Factor Questionnaire (16PF) which address the use of the tests in a business rather than an educational or clinical setting, and where adequate data are presented. For the CPI, 9.9 per cent of correlations (that is, for two of the 20 scales) were 'significant', and for the 16PF, 7.3 per cent (that is for one of the 16 scales).

These results suppose that the most

distribution of (ordered) correlations using the method of rankits. Ordering observed correlations in the same way, we can gain an impression of the extent to which observed correlations depart from what might be expected by chance alone. Table 1 gives an example from a real study of 38 staff supervisors.

The first three observed correlations are 'significant' at the 0.05 level, but clearly the two distributions are as close as makes no difference. Yet on the basis of such data, claims are made for the predictive validity of tests.

The basic concern of those who would use personality tests is usually whether one can have confidence that predictive relationships between test scores and criteria would recur in a cross-validation. In other words, has a real underlying relationship been identified? Going for the largest correlations looks the safest route. But what does one expect the largest correlations to be? Not zero, even on a null hypothesis of zero underlying correlations. Assume 30 test scales, a single criterion and a sample of 50. Ignoring sign (because we are talking about fishing trips with no hypothesized direction for relationships), the expected value of the smallest correlation is never zero, and the expected value of the largest is 0.34. The 95th percentile point of the distribution of the largest correlation in a set of 30, again ignoring sign, is about 0.42.

Looking at correlations in order of magnitude introduces a further complication in that under the null hypothesis of zero correlations in the population, expected values are not independent. In fact

they are positively correlated, implying that one chance high positive correlation is likely to be accompanied by others. This is independent of any associations attributable to correlations amongst test scales (and is believed by writers of textbooks — for example, H.A. David (*Order Statistics*; Wiley, 1981) — to be intuitively obvious).

To be concrete, there is little evidence of enduring relationships between personality test scores and measures of success at work. For instance, Table 2 shows a typical case of results failing to cross-validate, extracted from the validity review mentioned earlier. The test has 30 scores, of which 21 showed no 'significant' correlations with the extent to which sales targets were met.

So these sales managers did better if they were indecisive in 1985, but this did not carry over into 1986, though it began to be important to lose emotional control as that year wore on, particularly if optimism took hold, critical attitudes prevailed and modesty were out of the window. This is of course nonsense. The truth of the matter is that these correlations, despite their stars, are well within the bounds of what chance might throw up.

There are more sophisticated forms of validity delusions. Readily available statistical software allows easy computation of multiple correlations, which may lead to the claim that as a set the scales predict job performance, in accordance with a linear regression equation. And indeed, large multiple correlations are frequently found. But large numbers of predictors in combination with small sample sizes inevitably yield large multiple correlations. For example, when population correlations are all zero, the expected value of the multiple correlation coefficient between 30 predictors and one criterion on a sample of 50 subjects is 0.77. What psychologist would not crack a bottle of bubbly on the strength of such a result?

We are not suggesting that personality tests have no uses, or that there are no stable underlying aspects of temperament which are important in the determination of behaviour. Indeed, for counselling purposes, or in other situations where self perception is as important as the truth, they may be invaluable. But we see precious little evidence that even the best personality tests predict job performance, and a good deal of evidence of poorly understood statistical methods being pressed into service to buttress shaky claims. If this is so for the most reputable tests in the hands of specialists, one may imagine what travesties are committed further down market. But we leave this as an exercise for the reader. □

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TABLE 2 Observed correlations between test scores and sales performance over time

Test score	1985 Sales target	1986 (first half) Sales target	1986 (second half) Sales target
Persuasive	—	0.25*	0.35*
Outgoing	-0.23*	—	—
Affiliative	0.26*	—	—
Modest	—	—	-0.39**
Emotional control	—	—	-0.29*
Optimistic	—	—	0.28*
Critical	—	—	0.29*
Achieving	—	—	0.32*
Decisive	-0.27*	—	—
	(n=88)	(n=91)	(n=56)

Endothelins come home to roost

The reports elsewhere in this issue of the structure of two receptors for the potentially vasoconstrictive endothelins are a reminder of the power molecular biology is bringing to pharmacology (and much else besides).

CELL membrane receptors transmit specific messages to alter the function of a cell. On pages 730 and 732 of this issue^{1,2}, two independent Japanese groups describe the structure of two different receptors for the family of peptides known as endothelins, the most powerful of all substances in raising the blood pressure and closing down the circulation.

The endothelin story is remarkable. Highsmith and his colleagues³ found that endothelial cells in culture elaborate into their medium a peptide vasoconstrictor substance. In searching for a topic for his PhD thesis, Masashi Yanagisawa, with Hiroki Kurihara, found Highsmith's papers and suggested to his supervisor at Tsukuba University, Tomoh Masaki, that he should take up the problem. Masaki agreed and, as the project developed, brought a sizeable team into the work, including not only Yanagisawa as the molecular biologist but also Katsutoshi Goto as the pharmacologist and Sadao Kimura as the biochemist. The result was that endothelin exploded into our consciousness in a paper published in *Nature*⁴ on 30 March 1988. It was so complete and thorough that one of my colleagues, who read it at the weekend, was convinced that it must have been intended by the editors as an elaborate scientific April fool's joke.

Endothelin (ET) is a 21-amino-acid peptide made by the endothelial cell from pre-pro endothelin (200 amino acids) and pro endothelin (38 amino acids; called 'big ET') by an unusual cleavage performed by an endothelin converting enzyme (ECE). The structure of ET-1 is twisted into a conical spiral by two disulphide bridges and it is the most potent vasopressor compound yet discovered, easily beating the previous record holder, angiotensin II, by some tenfold. What is more, the pressor effects of ET-1 are long-lasting, so that a single small injection into the circulation of a rat increases the blood pressure for an hour or more. Clearly, the endothelial cells lining the blood vessels would secrete a substance so potentially active on vascular smooth muscle only for some crucial physiological (or pathological?) purpose. Just as clearly, we do not yet understand how endothelin earns its living.

All the classical hormones were discovered by bioassay of active principles in extracts of different tissues. Only later did the chemists and biochemists come along to tell us the actual chemical structures

and the biosynthetic route by which the compound was made. Not so with the endothelins. A few months after the first publication, a second isopeptide was identified, initially called rat endothelin. With the advent of the third⁵, Masaki's team disclosed the existence of all three peptides (now called ET-1, ET-2 and ET-3) in man and other species. They differ from each other by a few amino acids, but quite enough to give them different properties. Interestingly, they also have high homology with the sarafotoxins found in the venom of the Israeli burrowing asp, suggesting that they have a long genealogical history.

Endothelin-1 is the only one of the three made by the endothelial cell. Its action as a vasoconstrictor and other effects suggest that it is designed to function as a local hormone, released by the endothelial cell to contract the underlying vascular smooth muscle. Fitting with this hypothesis, ET-1 is rapidly removed from the blood. Circulating ET-1 also releases the potent vasodilators prostacyclin and endothelin-derived relaxing factor (EDRF) from endothelial cells, thereby limiting its own vasoconstrictor effects⁶. The peptide also has proliferative effects, for instance on mesangial cells of the kidney⁶. Remarkably, and because of the techniques by which the endothelins were discovered, we do not yet know which cells make endothelins 2 and 3, although their distribution is quite widespread.

Last week, the Second International Conference on Endothelins was held in their home in Tsukuba City, Japan. Despite burgeoning interest (there were almost 400 participants) the functions of the endothelins are still far from clear, although many expect excess ET-1 to be linked with hypertension, heart attacks and similar conditions. As with other hormones, their precise effects will become clearer only when receptor antagonists or ECE inhibitors are available. Pharmaceutical companies, racing each other to find such potentially therapeutic agents, are keeping their compounds close to their chests and none was reported at the Tsukuba meeting. However, a neutral protease inhibitor, phosphoramidon, not only prevents the conversion of big endothelin to ET-1 but also prevents the rise in blood pressure induced by injections of big endothelin in rats. Excitingly, this crude inhibitor of ECE also gradually lowers the blood pressure of spontaneously

hypertensive rats⁷.

The two distinct receptors described^{1,2} probably serve different functions. Each belongs to the superfamily of rhodopsin-like receptors, with seven transmembrane domains. Each is coupled to a G protein. One¹ shows high specificity for ET-1 and the messenger RNA is widely distributed in the central nervous system, the heart and the lungs. Might this be the vascular smooth muscle receptor? The other² equally accepts all three endothelins, as well as sarafotoxins, and is coupled through a G protein to phospholipase C, leading to transient increases in intracellular free Ca²⁺. The messenger RNA is not found in vascular smooth muscle. Such characteristics suggest it may (amongst others) be the endothelin receptor on endothelial cells responsible for the release of prostacyclin and EDRF. A receptor nomenclature subcommittee of IUPHAR met in Tsukuba City and will recommend that the specific receptor¹ is called ET_A and the non-selective one² ET_B.

These two groups were lucky not only to submit their manuscripts for publication within two days of each other, but also to end up cloning two different endothelin receptors. Let us hope that the next groups will be just as lucky in identifying the other receptors involved.

At a ceremony associated with the Tsukuba City meeting, Masaki and his colleagues were awarded the second Tsukuba Prize consisting of a huge silver medal and a research grant of ¥5 million (£20,000). This recognition of a truly outstanding discovery will surely not be the last to do with the endothelins. Oh yes, I should mention that Yanagisawa was awarded his doctorate.

John Vane

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Transporters of delight

Peter Parham

THE immunological careers of class I major histocompatibility complex (MHC) molecules depend upon intracellular supplies of short peptides. Assembly, transport to the plasma membrane and molecular longevity of class I molecules are all enhanced by binding the right peptide. The discovery that peptides bound by class I molecules frequently derive from cytoplasmic proteins suggested that active transmembrane transport of peptides from the cytoplasm to the endoplasmic reticulum occurs. Further support for this idea came from characterization of mutant cell lines which inefficiently assemble class I molecules and have properties consistent with defects in peptide transport. Since elaboration of this model (see figure) by Townsend *et al.*¹, there has been a concerted effort to find evidence for the putative peptide transporters. The search was first narrowed by finding that the genes which control class I assembly map to the MHC^{2,3}. Four papers, one in this week's *Science*⁴ and three in this issue⁵⁻⁷, now describe the cloning and sequencing of 'novel' MHC genes and their endorsement as candidates for peptide transporters. The credentials of these candidates look impeccable — appropriate positions in the MHC and membership of the 'ABC' superfamily of transmembrane transporters — but their performance on the job is as yet untested.

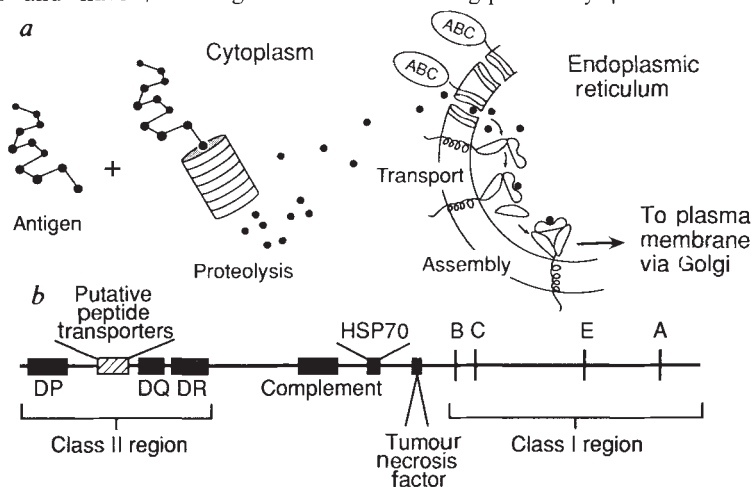
It was indeed fortunate that genes affecting class I assembly mapped to the MHC^{2,3}. This region of mammalian chromosome, about 500 megabases in size, has for many years been the site of intensive immunogenetic investigation and many of its genes have been identified and ordered. Most of the known gene products are implicated in the workings of the immune system and for many reasons immunologists continue to fish this deep MHC for undiscovered genes. Although the results and message of the four papers are similar, the circumstances by which various groups became poised to sight the peptide transporters were rather different.

For almost a decade Monaco has studied an enigmatic intracellular protein complex of 16 polypeptides, each of relative molecular mass 15,000–30,000 (M_r 15–30K) (refs 8 and 9). The complex (M_r 580K) is immunoprecipitated with allo-

antisera raised against products of the murine MHC (H-2), indicating that at least one of the component low-molecular-weight proteins (LMP) is polymorphic and encoded by an MHC gene. Monaco *et al.*⁴ have now narrowed the source of such genes to the class II region of H-2 and report on a 'fishing expedition' targeted to the segment between the $A_{\beta 2}$ and $A_{\beta 3}$ genes. While fishing for LMP genes (still to be landed and further discussed below) they came across two previously undiscovered homologous genes which they call *HAM1* and *HAM2* (for histocompatibility antigen modifier). The *HAM* genes resemble nothing previously

Butcher and their colleagues, who have independently identified the rat homologues of the *HAM* genes and associate expression of these genes with differences in conformation and antigen presentation of a class I MHC molecule (ref. 5, on page 738 of this issue). Their story began in 1983 when Livingstone *et al.*¹² reported that "Recombination within the MHC can change the specificity of class I major transplantation antigens", a phenomenon they subsequently ascribed to a polymorphic class I modification locus (*cim*), within the class II region of the rat MHC (*RTIA*). Alleles of this locus affect the recognition of the RTIAa class I molecule by both antibodies and cytotoxic T cells¹³. To clone transcripts derived from *cim* genes, Deverson *et al.*⁵ obtained cosmid clones covering the *cim* region and with this bait caught two "relatively abundant" clones from a library of complementary DNA. By sequence comparison these genes, designated *mtp 1* and *mtp 2* (MHC-linked transporter protein), are the rat homologues of *HAM1* and *HAM2* and are judged by the authors to be "attractive candidates for the hypothetical class I peptide transporter".

The investigations of Monaco *et al.* and Deverson *et al.* were guided by their respective discoveries of unexpected protein spots and curious T-cell reactivities. In contrast, those of Trowsdale *et al.* (ref. 6, page 741) and Spies *et al.* (ref. 7, page 744), which lay



a, Degradation of a cytoplasmic protein antigen into peptides (●), their transport from cytoplasm to the endoplasmic reticulum and assembly with class I MHC molecules. b, The human MHC (HLA complex) and the position of the genes encoding the putative peptide transporters. (After refs 15 and 16.)

extracted from the MHC and encode proteins similar in size, sequence and structure to members of a large family of proteins that transport all manner of molecular species across cellular membranes^{10,11}.

This family has mammalian, yeast and prokaryotic members, and includes the cystic fibrosis gene. Key features are a conserved cytoplasmic ATP-binding domain of about 250 residues and an N-terminal domain containing several hydrophobic membrane-spanning sequences. Proteins in this ABC (ATP-binding cassette) superfamily of transporters carry ions, small molecules and, more importantly for this discussion, peptides^{10,11}. On the basis of this potent, but circumstantial, evidence Monaco *et al.* postulate that these MHC-located ABC transporter genes "may be involved in transporting antigen or peptide fragments thereof, from the cytoplasm into a membrane bounded compartment containing newly synthesized MHC molecules".

Increased confidence in this interpretation comes from the studies of Howard,

claim to the human peptide transporters, are founded in the systematic trawling of unexplored regions of the HLA complex for undiscovered genes. Trowsdale's team has isolated "novel genes in the class II region of the human MHC" by fishing from the CpG islands that are often found near the 5' ends of genes. The *RING4* gene maps between the *DOB* and *DNA* genes (see figure), an HLA region that probably corresponds to *cim* of the *RTI* complex, and sequence determination confirms its membership in the ABC superfamily of transporters. Monaco *et al.* present a partial amino-acid sequence of *HAM2*, and comparison of the *HAM1*, *HAM2* and *RING4* sequence in this segment favours *RING4* as the human homologue of *HAM1*. Trowsdale's promise of a second *RING4*-like gene suggests there is also a human homologue for *HAM2*.

Spies and colleagues tell a similar tale — good evidence for one transporter gene and a mention in passing of another. To do this Spies, who has charted large segments of the HLA region¹⁴, linked forces with DeMars and Pious, specialists in produc-

tion and analysis of HLA mutant lymphoblastoid cell lines. By deletion mapping of informative mutants and chromosome-walking they identified a transporter gene they call *PSF(Y3)* (peptide supply factor) and which from its partial amino-acid sequence is identical to *RING4*.

Despite the diversity in nomenclature — *HAM*, *mip*, *cim*, *RING*, *PSF* — there is welcome uniformity in the results and interpretation of the four papers. Collectively they argue for ATP-dependent peptide pumps which supply nascent class I MHC molecules with peptides, and thereby fuel the endogenous pathway of antigen presentation. It is, however, an irony of the 'clone-and-sequence' approach that the information to be obtained from the sequence of a new gene often inversely correlates with the novelty of its sequence. Certainly this is true for the predicted protein sequences described in these four reports, for without their obvious similarity to well-characterized transport proteins^{10,11} the case for involvement in antigen processing and presentation would be nowhere near so persuasive. In fact, and as the authors point out, none of the papers report on the expression of the encoded proteins, their function or their capacity to remedy the defect in mutant cells deficient in class I assembly. To some readers this might seem a bit fishy, but the absence of such experiments may merely reflect the accelerating pace of research.

It now seems that many MHC genes are involved in distinctive aspects of antigen processing and presentation. To the class I and class II antigen-presenting molecules are added cytokines¹⁵ and chaperonins^{16,17} and now peptide transporters. Furthermore, there is circumstantial evidence that proteases will also be mapped to the MHC in the guise of Monaco's MHC-linked *LMP* genes^{8,9}. The striking proper-

ties of the LMP complex are mimicked in Macropain, a large intracellular ATP-dependent protease comprising 13 polypeptides of *M_r* 20–35K ordered into a cylindrical structure of *M_r* 650K (refs 18 and 19). The similarities could be fortuitous, but I doubt it and suggest that the LMP particle may be a Macropain-related protease involved in the processing of antigens. Consistent with this role for the LMP complex is the increased expression of LMP proteins in the presence of γ -interferon⁸, a property shared both by class I and II MHC molecules and the COSMOLOGY

putative peptide transporter genes⁶. In addition these genes all exhibit polymorphism and, to extend the arguments of Deverson *et al.* and Spies *et al.*, their MHC linkage could facilitate evolution of MHC haplotypes encoding proteases, transporters, and presenting molecules with compatible peptide specificities that offer advantage in responding to antigens of pathogenic origin. □

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Einstein's blunder resurrected

Edmund Bertschinger

WAS the cosmological constant really Einstein's biggest blunder? Maybe not, if some new astronomical observations and their interpretations are correct. The cosmological constant Λ was originally added to the field equations of general relativity to reconcile the theory with the belief that the Universe is quasi-static: without it, gravity unopposed would cause the Universe to collapse. After the discovery that the Universe is expanding, cosmologists generally have dismissed the possibility of a nonzero Λ . With increasing frequency, however, new observations are challenging the standard cosmological theories with $\Lambda = 0$. On page 705 of this issue¹, Efstathiou, Sutherland and Maddox present what may be the strongest case to date for a nonzero cosmological constant.

During the past decade, the standard Big Bang model of cosmology has been refined with the addition of two key ingredients. The first is the idea that an early stage of exponential expansion² inflated a microscopically tiny volume to a size much larger than the presently observable part of the Universe. The inflationary expansion stretches the geometry of spacetime so much that the residual gravitational curvature of space should be negligibly small today. In the absence of a cosmological constant, the implication is that the mean mass density Ω_0 , in units of the 'critical' density that defines a flat universe, should equal one. Ironically, the exponential expansion is thought to have been driven by a temporary field that was equivalent to a nonzero cosmological constant ($\Lambda = 8\pi G\rho_\phi$, G being Newton's constant and ρ_ϕ the energy of this field). Inflation does not preclude the possibility of a residual cosmological constant today³, provided that it satisfies the relation $\Omega_0 + \Lambda/3H_0^2 = 1$, where H_0 , the Hubble parameter, is the rate at which the Universe is currently expanding.

If the inflationary paradigm is correct and $\Lambda = 0$, ordinary matter including luminous stars and galaxies can contribute

only a small fraction (perhaps 5 per cent) of the total mass density required to make $\Omega_0 = 1$. The second ingredient added to the standard Big Bang model is, therefore, dark matter. Among theorists, the most popular form of dark matter consists of exotic new particles which have negligible thermal velocities after the Big Bang, generically called cold dark matter. A remarkably successful theory for describing how galaxies formed in the primordial Universe has been developed with cold dark matter^{4,5} in an inflationary universe with $\Omega_0 = 1$ and $\Lambda = 0$. However, this theory is currently facing a number of difficulties with recent observations, particularly with the distribution of galaxies on scales larger than 10 megaparsecs. (Within a group, galaxies are typically separated by less than 1 megaparsec.)

Efstathiou, Sutherland and Maddox now add to the list of difficulties. Their new results come from an analysis of galaxy clustering from their Automated Plate Measuring Galaxy Survey⁶. As have other groups, they find that the large-scale distribution of galaxies is less smooth than predicted by the $\Omega_0 = 1$, cold-dark-matter model. Furthermore, the cold-dark-matter model appears to be more consistent with the observed large-scale structure if Ω_0 is reduced to around 0.2 which has the effect of enhancing the density fluctuations on large scales relative to small scales. But on the largest scales, according to measurements of the microwave background radiation, the Universe is smooth, and it is this that motivates Efstathiou and colleagues to invoke the cosmological constant.

There are many other ways, even with $\Lambda = 0$, to increase the amplitude of large-scale structure while satisfying the upper limits on the anisotropy of the microwave background radiation. Hybrid inflationary models with hot and cold dark matter might work. Topological defects such as cosmic strings or textures might initiate large-scale structure efficiently. Or a

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simple upward revision of the (theoretically arbitrary) normalization of the overall density fluctuation amplitude in the cold-dark-matter model, combined with new mechanisms for suppressing small-scale structure, might lead to an acceptable model for the formation of both galaxies and large-scale structure.

But because the cosmological constant affects the overall geometry and expansion of the Universe, it should be possible to get more direct evidence of it. In particular, counts of the number of high-redshift (distant) galaxies provide a potentially strong indicator of the geometry provided that galaxy evolution can be understood sufficiently well. The dimensionless product of the age of the Universe (t) and the Hubble expansion parameter, provides the strongest test of all. The standard cosmological models with $\Lambda = 0$ all predict $t < H_0^{-1}$ ($t < 20 \times 10^9$ yr for $H_0 \geq 50$ km s⁻¹Mpc⁻¹) as gravity will have slowed down the initial expansion rate. If $\Omega_0 = 1$ and $\Lambda = 0$, the upper limit on t is reduced by a factor of two thirds. A positive cosmological constant increases the expansion rate for a given t , making it possible to have $H_0 t > 1$. If it can be shown convincingly that $H_0 t > 1$, the case for a cosmological constant would become strong.

A nonzero cosmological constant would have profound and, most physicists would add, disturbing implications for fundamental physics. A cosmological constant of the magnitude required to be cosmologically interesting (and not devastating) requires unnatural fine tuning of particle physics models by 120 orders of magnitude. No natural physical mechanism has been suggested which might lead to an appropriate nonzero Λ . When particle physicists speak of solving the cosmological constant problem⁷, they mean finding a mechanism that drives Λ exactly to zero. If astrophysicists conclude that it is not zero, the problem will be much worse.

Despite the strong reservations that most physicists and astrophysicists have about invoking a cosmological constant, its existence must ultimately be demonstrated or refuted empirically. Cosmologists must be prepared to dispense with their sacred theoretical cows. However, I, for one, feel a strong sense of unease in the present circumstances. Is Λ the joker in the cosmologist's deck of cards, waiting to be drawn when needed? □

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Around Venus in 37 days

William B. McKinnon

MAGELLAN, NASA's space probe that has orbited Venus since mid-August, has been passing back startling synthetic radar images of the planet — huge impact craters surrounded by fantastic lobate ejecta blankets, giant volcanic calderas and mountain ranges that boggle the mind. The first detailed analyses of data from the first 37 days of the mission's initial 243-day mapping cycle were presented at the American Geophysical Union Fall Meeting in San Francisco two weeks ago. The general reaction to the presentations, both oral reports by Magellan Science Team members and detailed poster displays, was stupefaction. No individual can keep up with the flood of information, and one team member remarked during his talk that even he had not seen the image he was about to show. Nevertheless, tentative understandings of (or at least approaches to understanding) our sister planet were discussed.

Radar images of venusian impact craters are provocative, partly because impact morphology is largely defined, so any deviation from planetary 'norms' is intriguing, but also because of the inherent beauty of their symmetry and radar contrast. Venus's mostly volcanic surface is relatively dark to Magellan's side-looking radar, which means that the surface is relatively smooth as seen by the radar's 12.6-cm wavelength. The crater ejecta and floor material are far rougher, and therefore brighter to the radar, perhaps owing to the increased block content or an abundance of solidified impact melt. The larger craters are lovelier still, flooded with radar-dark material, presumably lavas erupted from below.

The influence of the atmosphere on impactor break-up and crater formation is clear. According to Roger Phillips (Southern Methodist University), there are no identified single, primary impact craters less than 3-km across, and in a limited size range above this threshold, the impacts are irregular in shape, have multiple floors

divided by septa, or form clusters or even discrete fields of smaller craters. These morphologies were all predicted theoretically and further data may allow the identification of different classes of impactors, that is, metal and rock asteroids and icy comets, which have different maximum diameters for atmospheric breakup, owing to their widely differing mechanical strengths.

The average crater density of the surface explored so far, according to Phillips, indicates that, on average, the surface is about 400 million years old (with a factor of two uncertainty due to different estimates of the impactor flux at Venus). This is relatively youthful by planetary standards, and there are also regions devoid of craters, regions whose size makes this absence statistically significant. The implication is that Venus is likely to be volcanically active today, and that major episodes of volcanic burial may be a dominant mechanism in erasing craters.

Actually, it is not entirely clear how craters are lost on Venus. The absence of both a hydrological cycle and small-scale bombardment eliminates the two main mechanisms by which land forms are degraded elsewhere in the Solar System. Buried beneath a thick atmosphere that is equivalent in mass to 1 km of water, the craters might as well be buried at sea. From the Magellan data acquired so far, they seem to have aged little, if at all. Volcanism (or tectonic activity) may be the mechanism for crater removal, but Phillips noted only two craters caught in the act of volcanic burial, hence the inference of past episodes of massive (that is, complete) basalt flooding.

There may be some evidence of crater ageing. Raymond Arvidson (Washington University) reported the discovery of large horseshoe-shaped surface features that appear dark in both radar and in brightness-temperature (emissivity) maps. The features open to the west, the dominant direction of Venus's high-altitude winds,

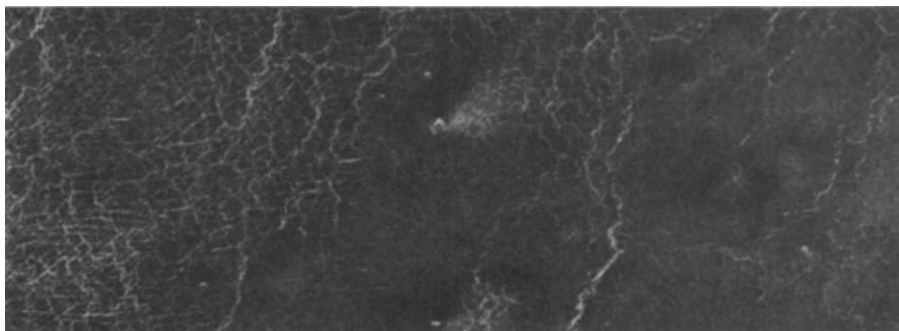


FIG. 1 Region of small volcanoes whose dark deposits may bury pre-existing fractured plains. The deposit is diffuse (it feathers out into the plains) and lacks what would be interpreted on the Earth and Mars as wind shadows. The larger, central volcanic crater is 1 km in diameter.

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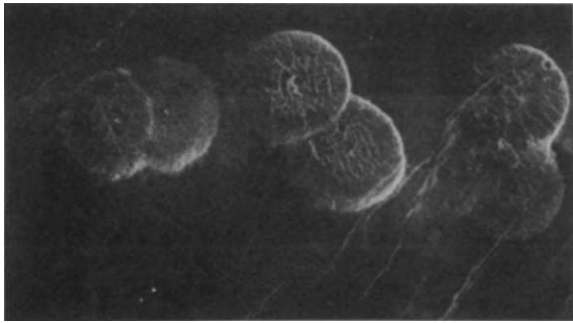


FIG. 2 Seven circular domical hills averaging 25 km in diameter with maximum heights of 750 m. These are probably eruptions from central vents of relatively viscous lavas, with 'chilled' (as chilled as anything gets on Venus) top surfaces stretched and cracked during flow.

typically stretching over 1,000 km. At the base of each shoe is an impact crater. The speculation is that ejecta launched high-enough (or material ablated from the impactor as it raced through the atmosphere) is entrained in the zonal wind, spreading and eventually dropping out to the west. Relatively few impacts are associated with these features, hence the real excitement. Through chemical weath-



FIG. 3 The southern extent of the elevated plateau Lakshmi Planum. The image is approximately 540 by 275 km. The plateau is covered by relatively homogeneous volcanic deposits, and is rimmed to the south by the Danu Montes mountain belt. The mountains rise 500 m above the plateau but drop very sharply (by around 3 km) to the radar-dark plains at the bottom of the image. Also seen are a 14-km diameter crater on the plateau, and a circular structure at the bottom which may be an impact crater now being tectonically and volcanically obliterated.

ering and other sedimentological processes, these features may disappear with time; only the youngest craters may have them. Thus the beginnings of an impact stratigraphic timescale may be defined, with these broad features providing the marker horizons to establish the relative ages of volcanic and tectonic events.

Arvidson also described evidence of surface-atmosphere interactions such as wind streaks and probable dune fields associated with crater ejecta. Erosion is also indicated by exposures of fractured plains in the wind shadows of small volcanoes (Fig. 1). That some volcanoes are surrounded by diffuse radar dark deposits may be evidence of pyroclastic volcanism. Given the high atmospheric pressure on the planet, this argues for a significant volatile content in the Venus mantle (perhaps CO_2).

Volcanism is otherwise the dominant surface process, and J.W. Head (Brown University) described the wide variety of volcanic landforms. These include shield volcanoes with associated collapse calderas, pancake-like viscous domes (Fig. 2), lava plains and rivers, and highland plateaus (Fig. 3). The volcanic and tectonic interactions of venusian coronae (large oval features hundreds of kilometres in diameter, with associated caldera-like inner structures and related flow units) were clarified. Patterns of extensional faults radiate from the high-standing centres of many coronae, and are ringed by compressional ridges and an outer trough. The extension and volcanism are related to the ascent and partial melting of hot mantle material. Models were presented that account for all of the tectonic features by viscously coupling the flow associated with mantle plumes or hotspots to the lithosphere.

The interaction between the mantle and lithosphere lies at the heart of elucidating Venus's evolution. Venus tectonics, according to Sean Solomon (Massachusetts Institute of Technology), are remarkably like those on Earth in some ways. Compressional mountain building occurs on the planet (Figs 3 and 4), and offers the only clear analogue to mountain building on the Earth. Whether this analogy can be carried all the way to plate tectonics is not yet clear. Solomon noted

that although strike-slip displacements have been identified, no large offsets, characteristic of rigid terrestrial plate boundaries, have been found. But it is far too early to rule out plate tectonics on Venus, because the regional picture is unclear, and the large highland region, Aphrodite Terra, possibly related to crustal spreading, has not been imaged. What is clear is that Venus's lithosphere and crust respond readily to forces from the interior. There are regions of extension being ripped apart, zones of faulting indicating limited distributed shear, and regions elevated by multiple thrusts (Fig. 4). The steepness of some compressional fronts (essentially at the angle of repose) implies that mountain building continues today. Other highland regions are broken up by normal faults and graben (Fig. 4), perhaps caused by gravitationally driven collapse (as occurs in the Himalayas).

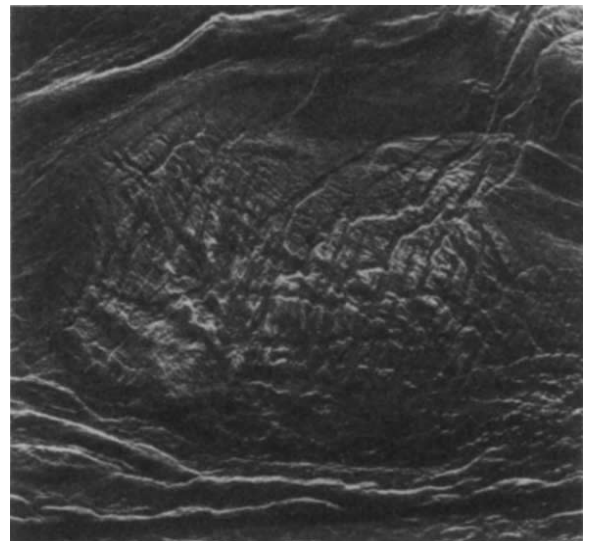


FIG. 4 Juxtaposition of compressional and extensional tectonics. The long bright features are part of the Freyja Montes mountain belt, interpreted to be created from multiple overlapping thrust sheets. The elevated, highly faulted block in the centre, about 70 by 125 km, seems to be spreading under its own weight in a different direction from the thrust. In the absence of dynamic compressive support, thicker crust on Venus may be too weak to resist gravity, because of high surface temperatures and Earth-like thermal gradients.

If subduction does not occur, then gravitational spreading may be the penultimate fate of crust on Venus. Its ultimate fate may be burial by new volcanics. Solomon noted that the oldest crust in the lowland plains are the slightly high-standing tesserae. These regions have been tortured by multiple episodes of tectonic deformation. Other schemes for crustal recycling exist, and plate tectonics could incorporate large amounts of ductile strain. This will be the ultimate result from the Magellan mission: a broader understanding of how Earth-like planets behave. □

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Some signal developments

Henry R. Bourne, Lisa Wrischnik and Cynthia Kenyon

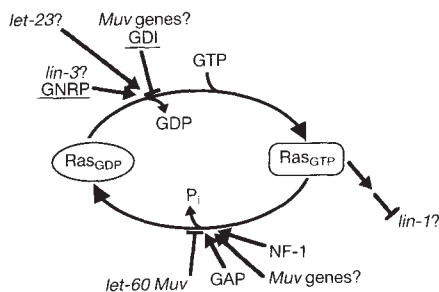
WHAT molecular signalling machines tell a precursor cell to develop into a specialized structure? In one case, described in three papers¹⁻³, including that by Aroian *et al.* on page 693 of this issue¹, these machines turn out to be a receptor tyrosine kinase¹ and a *ras* protein^{2,3}.

The vulva of the tiny nematode worm, *Caenorhabditis elegans*, was until recently no more than *cet obscur objet du désir* for other nematodes. Now the newly identified proteins that signal development of this specialized epidermal structure promise to make it a beacon for developmental biologists. These two signalling machines, familiar from appearances on other stages — cell proliferation and cancer, among others — provide tools for teasing out the secrets of vulval development. The study of vulval development, in turn, may add to the cast of characters known to interact with these signalling proteins.

In normal development, only three of the six vulval precursor cells of *C. elegans* contribute to formation of the mature vulva, whereas the other three become part of the epidermis. A cell that lies above the vulval precursor cells, known as the anchor cell, sends an inductive signal to the three that will form the vulva. This positive signalling is necessary to overcome an inhibitory signal sent to the precursor cells by the surrounding epidermis⁴. Recessive mutations have revealed candidates for both their negative and their positive regulation. For example, mutations that inactivate the *lin-15* gene abrogate a negative control, causing all six vulval precursor cells to follow the vulval developmental pathway and producing the Muv (multiple vulvas) phenotype. Animals homozygous for loss-of-function mutations in the *let-23*, *let-60* or *lin-3* genes exhibit the opposite phenotype, called Vulvaless (Vul), in which all six precursor cells enter the epidermal pathway and no vulva develops.

Reports from Sternberg and his colleagues^{1,3} now reveal familiar proteins behind the enigmatic codenames for *let-60* and *let-23*. Chromosome mapping, DNA-mediated transformation and sequencing of a point mutation revealed that *let-60* encodes a 184-residue protein, identical in 84 per cent of its first 164 amino acids to the *ras* gene products of rat, human and *Drosophila*⁵. Chromosome mapping of *let-23* to a region containing a viral tyrosine kinase gene homologue led to the discovery that the *let-23* gene encodes a tyrosine kinase with features that place it in the epidermal growth factor receptor subfamily¹. These genes may mediate the response of vulval precursor cells to extrinsic signals.

The two signalling machines relate to one another in *C. elegans* much as they do in mammalian cells. Increased expression of the *ras* protein (from multiple copies of exogenous *let-60* DNA) suppresses the *let-23* Vul phenotype³, showing that the receptor tyrosine kinase acts upstream of the *ras* protein in promoting vulval development. This class of receptors acts upstream of *ras* in promoting proliferation of mammalian cells (reviewed in ref. 5); moreover, ligands for these receptors —



The *ras* protein in *Caenorhabditis elegans*. Shown is the cycle of the protein between an inactive GDP-bound form and an active GTP-bound form.

for example platelet-derived growth factor (PDGF) — can stimulate formation of the active GTP-bound form of p21^{ras} in mammalian cells⁶.

Like other GTPases⁷, mammalian and yeast *ras* proteins cycle between an inactive GDP-bound form and an active GTP-bound form. This cycle (see figure) helps us to interpret Vul and Muv phenotypes produced by *let-60* and other mutations and suggests strategies for identifying other proteins in the regulatory pathway. The cycle is regulated by the rates of two reactions, dissociation of GDP and hydrolysis of GTP; these reactions increase and decrease, respectively, the fraction of *ras* protein in the GTP-bound form. The first reaction is stimulated by a guanine nucleotide releasing protein (GNRP) and — in the case of certain *ras*-like GTPases — is slowed by an inhibitor of GDP release (GDI). The *ras* GTPase reaction is markedly accelerated by two previously identified proteins, one called GTPase activating protein (GAP), and the other encoded by the neurofibromatosis-1 (NF-1) gene⁸⁻¹⁰.

Beitel *et al.*² identified the altered nucleotides in five independent dominant-positive ('gain-of-function', or *gf*) *let-60* Muv mutants. These were reassuringly familiar: all five mutations substituted glutamate for glycine at position 13, where similar substitutions produce increased activity of mammalian p21^{ras}, resulting in neoplastic transformation¹¹. Such *gf* mutations in p21^{ras} increase the

protein's activity by preventing GAP-induced acceleration of GTP hydrolysis⁵, implying that the *let-60* protein is also active in its GTP-bound form.

If so, our knowledge of proteins that regulate the mammalian and yeast counterparts of *let-60 ras* suggests that there are other genes that should affect vulval development. Thus, genetic inactivation of the appropriate GNRP should produce a recessive Vul phenotype. Because the postulated GNRP acts upstream of the *let-60 ras* protein, the phenotype produced by its genetic inactivation would not be detected in animals carrying *gf let-60 ras* mutations. The *lin-3* gene product, thought to act upstream of *let-60*¹², could turn out to be a GNRP for *let-60 ras* protein. Reasoning in the reverse direction, we predict that genetic inactivation in *C. elegans* of negative regulators such as GDI, GAP or NF-1 will produce recessive Muv phenotypes that require the presence of a normal *let-60 ras* protein. Several Muv mutations have already been found, and the genes they define may be these very regulators.

As well as cloning and sequencing a gold-mine of over 20 genes already known to affect vulval development, investigators will use specific *let-60* mutations as tools in searching for other elements of the signalling pathway. One strategy for finding downstream genes in the pathway that promotes vulval differentiation would be a deliberate search for Vul mutations that prevent the Muv phenotype produced by *gf* mutations in *let-60 ras*. Another is to search for mutations that suppress the loss-of-function *let-60* Vul phenotype; recessive mutations in *lin-1* have just this property. Such searches could even uncover the effector protein that is activated by the *ras* protein, analogous to adenyl cyclase in *Saccharomyces cerevisiae*. This would be a major achievement, because considerable effort has failed to identify the corresponding *ras* effector in mammalian cells (for reviews see refs 5, 7, 13).

Several *let-60* mutations produce a dominant-negative Vul phenotype^{2,3} — that is, these mutations prevent vulval development even in the presence of a normal *let-60* allele. Mutations in other genes that prevent these phenotypes could teach us a great deal. Precedents in other *ras* proteins¹⁴ and in another GTPase, bacterial elongation factor Tu¹⁵, show that dominant-negative mutant GTPases often prevent activation of their normal counterparts by binding to and sequestering the GNRP; consequently, the dominant-negative phenotype can be prevented by increasing expression of the GNRP^{14,15}. Thus a mutation that induces increased expression of the appropriate GNRP might overcome a dominant-negative *let-60* Vul phenotype. In addition, a search for Muv phenotypes in animals

expressing a dominant-negative *let-60* allele could lead to discovery of *gf* mutations in genes for proteins that act downstream of *let-60 ras* in vulval differentiation.

Beitel *et al.*² also sequenced eight independent *let-60* reduction-of-function mutations that produce a recessive Vul phenotype. The locations of amino acids substituted in such mutants should be of negligible interest, because mutations can disable a *ras* protein in many ways. But in fact the three-dimensional structure of p21^{ras} shows that five of these eight mutations are remarkably close to one another on the protein's surface. Of these five, four (including three substitutions of alanine at position 66, and a substitution for glycine at position 75) are located at either end of an α -helix whose conformation changes in response to binding of GTP^{16,18}. This proximity is not likely to have resulted from coincidence. Instead, it

probably reflects a selection procedure favouring mutant *let-60* alleles that retain partial function. All five of these recessive mutants were obtained in a procedure that required a large proportion of the mutant animals to reach a stage at which vulval differentiation can be scored — this stage is later than the point at which severe loss of *let-60* function kills most *C. elegans* larvae^{3,12,19,20}. Perhaps one of the simplest ways for a mutation to induce a partial loss of function is to impair interaction of *let-60 ras* protein with a specific protein; if so, the locations of the substituted amino acids indicate the binding site of the putative protein ligand, which might be a GNP or the elusive effector. □

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GREENHOUSE WARMING

Vegetation an unlikely answer

William H. Schlesinger

WHETHER land plants and soils can act as a net source or a sink for CO₂ is a critical and controversial issue for understanding the current increase in atmospheric CO₂ and the potential for greenhouse warming during the next century. Using the past as an indication of the future, Adams *et al.*, on page 711 of this issue¹, suggest that the terrestrial biosphere contained significantly less carbon during the last ice age than it does today. They base their analysis on their best estimates of the distribution of land vegetation at the last glacial maximum which they compared with maps of the present-day distribution. The increase in carbon storage is due to the regrowth of forests on areas formerly covered by glacial ice and to the accumulation of soil organic matter in peatlands. Thus, vegetation took up CO₂ as the temperature warmed. Over the same interval, the atmospheric CO₂ concentration increased² from 200 to 280 parts per million (p.p.m.). Adams *et al.* suggest that the current concentration of CO₂ (350 p.p.m.) might be even higher if it were not for the uptake by land vegetation.

The maps of the distribution of vegeta-

tion 18,000 years ago show that the area of desert was 83 per cent greater than today — a conclusion roughly in line with most studies of lake palynology and measurements of desert dust in the Vostok Antarctic ice core³. As forests and woodlands have expanded onto these lands during the past 10,000 years, they have also acted to remove CO₂ from the atmosphere and store carbon in organic matter. The total storage of carbon on land during the Holocene is large enough to have removed the entire atmospheric content of CO₂, but equilibrium concentrations of CO₂ are maintained by degassing of CO₂ from the oceans.

That land plants and soils take up CO₂ is also indicated by the recent model of Prentice and Fung⁴; however, their estimate of the net storage of carbon on land during the past 10,000 years is smaller, mainly because they assume an unrealistically small area of desert during the last glacial. More significantly, Prentice and Fung suggest that land vegetation could act as a large carbon sink in the potentially warmer climate of the future. The storage could exceed 2×10^{15} grams of carbon

a year (g C yr⁻¹), which would significantly slow the rate of greenhouse warming due to CO₂. Tans, Fung and Takahashi⁵ indicate that the terrestrial vegetation and soils must already be acting as a sink for CO₂, as the atmosphere does not contain as much of the fossil fuel CO₂ as it should, a result they obtain by way of a new estimate for the oceanic uptake of atmospheric CO₂ that is derived from human activities. Tans *et al.* suggest that the terrestrial storage of carbon was $2\text{--}3.4 \times 10^{15}$ g C yr⁻¹ in the mid 1980s.

Although all these analyses appear to show that the terrestrial biosphere may act as a sink for CO₂ in warmer climates, they differ in degree. The vegetation changes indicated by Adams *et al.* yield an average storage of only 0.13×10^{15} g C yr⁻¹ on land during the Holocene. I estimate⁶ that the maximum potential sink in soils is not now likely to exceed 0.4×10^{15} g C yr⁻¹. These values are much smaller than the accumulation rates needed to reduce the potential for global warming due to CO₂.

The analyses by Adams *et al.* and Prentice and Fung are based only on changes in the distribution of vegetation in response to past and future climates. They do not include stimulation of photosynthesis and carbon storage as a response to higher CO₂ concentrations in the atmosphere. Indeed, to achieve the storage of carbon required by the model of Tans *et al.* would seem to require a substantial CO₂ fertilization effect, when most field-fertilization studies show little^{7,8}. In most cases net primary production is quickly limited by the low supply of nutrients in soils.

The potential for the terrestrial biosphere to store carbon is also limited by degradation of natural vegetation and soils in response to human population growth and agricultural expansion, particularly in the tropics. Houghton⁹, Woodwell¹⁰ and their collaborators have shown that there is a significant net release of CO₂ to the atmosphere from biomass destruction. As the human population continues to grow exponentially, the potential for vegetation shifts and CO₂ fertilization to create a carbon sink on land may become largely academic. The Earth's surface will be too disturbed. □

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Getting the bird

BIRD attack on milk bottles delivered to the doorstep is an important risk factor for infection with *Campylobacter jejuni*, an enteric bacterial pathogen, according to a recent case-control study by J. P. Southern *et al.* published in *The Lancet* (336, 1425–1427; 1990). The study, which began after a reported four-fold increase in *Campylobacter* cases in the Bridgend area of south Wales last May, implicates magpies and jackdaws. The authors report a strong correlation between bird attack on bottles and incidence of human illness; although they did not carry out a microbiological investigation, *Campylobacter* have previously been isolated from both species of bird.

Hubble trouble

In trenchant style (beginning with a quote from the *Federalist Papers* on the capacity of wise men to indulge in controversy), A. Sandage and G. A. Tammann, two veterans of the search for Hubble's constant, deliver their latest findings in the *Astrophysical Journal* (365, 1–12, 1990). In our immediate vicinity, Hubble's constant (H_0), the rate at which the galaxies recede, is widely believed to be 50 km per second per megaparsec, at the low end of the empirical spectrum of values, which stretches up to 100 km s⁻¹ Mpc⁻¹. But our local group of galaxies has a peculiar velocity towards the giant Virgo cluster, which contaminates extrapolations of H_0 to the much-desired cosmic value. Sandage and Tammann compare the Virgo cluster directly with more remote clusters to establish the 'true' recession velocity of Virgo. They deduce that $H_0 = 50$ holds true cosmologically as well as locally — a result that will surprise neither their supporters nor members of the $H_0 = 100$ gang.

Mating games

WORK by C. L. Jackson and L. H. Hartwell shows that cells of the budding yeast *Saccharomyces cerevisiae* choose their mating partner according to the strength of a pheromone signal. Haploid yeast cells exist in two mating types, **a** and **α** , which can conjugate to form **a/ α** diploids. Conjugation requires arrest of the mitotic cycle, activation of several genes essential for cell fusion and karyogamy, and a number of morphological changes that align the two cells. These processes are all initiated by the binding of a pheromone to specific cell-surface receptors. Writing in *Molecular and Cellular Biology* (10, 2202–2213; 1990) and *Cell* (63, 1039–1051; 1990), Jackson and Hartwell show that individual **a** and **α** cells select as a partner the cell secreting the most pheromone. Perhaps other eukaryotes respond to external signals in the same way to cause directed cell movement or growth.

Thereby hangs a tail

Chris Paul

WITH our anthropocentric view of evolutionary 'progress', we naturally seek our most distant ancestors among the invertebrates. There is a consensus that echinoderms (sea urchins, starfish and their allies) are our closest relatives among non-chordate phyla, but there has been considerable resistance to the only consistent attempt to develop a detailed account of echinoderm–chordate relationships. This is the work of R. P. S.

porous structure called stereom and, typically, pentameral symmetry; and, finally, the carapoids (Fig. 1d–f), bizarre, extinct creatures with echinoderm-like stereom plates, but no trace of pentamerism. There is general agreement that carapoids form an evolutionary line distinct from that of typical pentameral echinoderms^{3,4}. But according to conventional wisdom (Fig. 2a) invertebrate chordates are more closely related to vertebrates than echi-

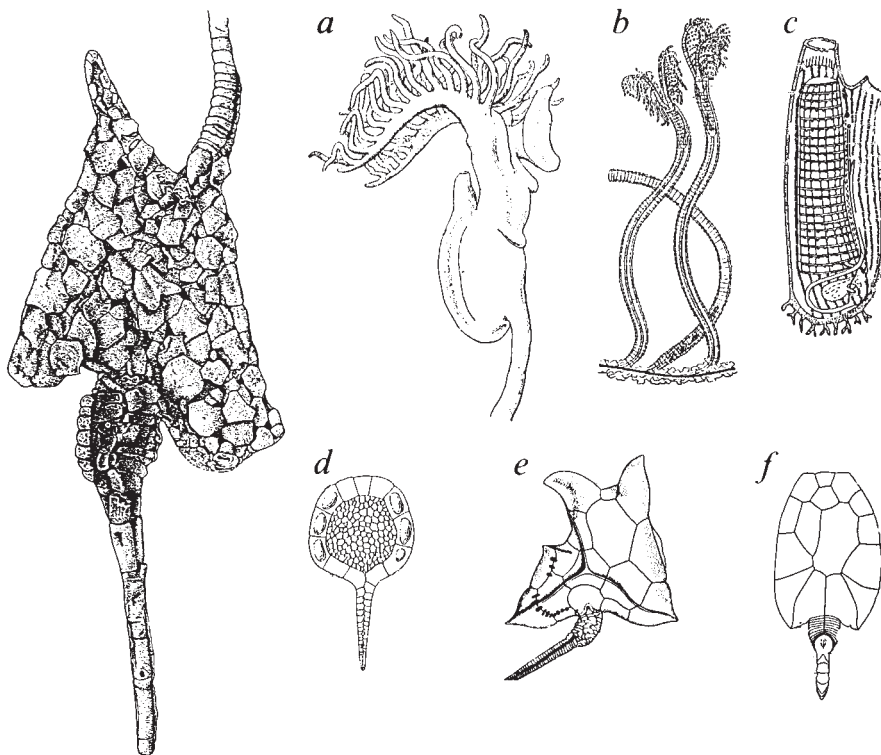


FIG. 1. Left, *Dendrocystoides scoticus* in dorsal view, the feeding arm being the longer structure at the top, the tail being below. Right, invertebrate chordates (a–c) and carapoids (d–f). a, b, *Rhabdopleura*, a hemichordate (a, an individual, b, the colony); c, *Ciona*, a solitary urochordate; d, *Trochocystites*, a cinctan; e, *Ceratocystis*, a cornute carpod; f, *Mitrocystella*, a mitrate.

Jefferies, whose latest contribution¹ deals with an obscure Palaeozoic fossil *Dendrocystoides scoticus* (Fig. 1). The paper depicts for the first time Jefferies's views of the relationships between all the groups of fossils that Jaekel² first distinguished as carapoids (and which most palaeontologists regard as aberrant echinoderms). It also confounds the alternative hypothesis about the carpod 'feeding tail' favoured by some students of fossil echinoderms.

The main controversy involves four groups of organisms. The vertebrates, a subgroup of chordates characterized by possession of a backbone made of vertebrae; the 'invertebrate chordates' (Fig. 1a–c), a morphologically diverse group of living organisms that at some stage in their life cycle possess a notochord which in vertebrates acts as the template for the backbone; the echinoderms, which lack a notochord but possess calcite plates with a

notochord, and carapoids are echinoderms because they possess stereom plates. Reduced to its simplest, Jefferies's view is that the phylogenetic relationships are more complex than that, with some carapoids being more closely related to vertebrates than to some invertebrate chordates (Fig. 2b).

During their ontogeny living echinoderms undergo remarkable, asymmetrical developments. Starting with an elongate, bilaterally symmetrical, motile larva with three pairs of coelomic cavities that are very similar to the larvae of some hemichordates, they develop extensive organ systems from the left-hand coeloms, whereas those on the right atrophy. Jefferies explains this by suggesting that an ancestor similar to the colonial hemichordate *Cephalodiscus* lay down on its right-hand side, a condition he calls dexio-

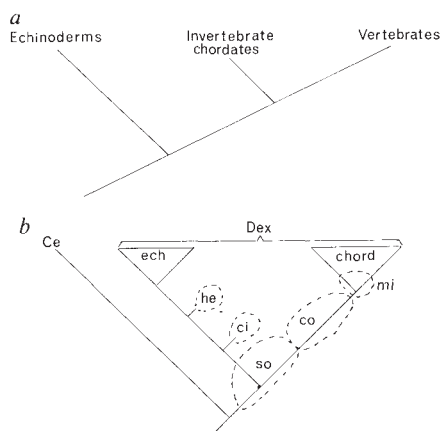


FIG. 2 *a*, The conventional view of echinoderm–chordate relationships. *b*, Jefferies's views. Ce, *Cephalodiscus*, a hemichordate; chord, crown-group chordates including urochordates, cephalochordates and vertebrates; ci, cinctans; co, cornutes; Dex, dextiothetes; ech, crown-group (radiate) echinoderms; he, helicoplacoids; mi, mitrates; so, solutes. (Modified from ref. 1.)

thetism. From this invertebrate chordate ancestor, echinoderms arose on the one hand, and chordates (including all the other invertebrate chordates) on the other. Furthermore, Jefferies thinks this fundamental division occurred within the carroids, with cinctans being stem echinoderms, and solutes, typified by *Dendrocystoides* being stem chordates. Personally, I think the fundamental division was between the radiate echinoderms and the carroids, and that the cinctan tail is homologous with the solute tail. But to me the evidence for dextiothetism seems incontrovertible.

All Jefferies's previous papers dealt with advanced carroids called cornutes and mitrates which possess a tail. Others have argued that this tail was a feeding organ⁵. Solute such as *Dendrocystoides* possess a tail and, opposite it, an arm which all workers agree was for food gathering. Jefferies convincingly demonstrates that the solute tail is homologous with that of cornutes. Because solutes possessed an anterior feeding arm, under the 'feeding tail' hypothesis the transition from solutes to cornutes requires transfer to food gathering at the back⁶, and is as unlikely as talking from the wrong end of the alimentary canal. Alternatively, one has to deny that solutes and cornutes are in any way related.

So how does one evaluate the rival hypotheses? The conventional view has the advantage of simplicity. Animals with a notochord are more closely related to us

than those without; stereom characterizes echinoderms. But it is incomplete and no proponent of it has produced a phylogeny based on detailed morphological comparisons showing even the relationships between the carroid groups, let alone relationships between invertebrate chordates and echinoderms. Denying relationships between solutes and cornutes

because of the needs of the feeding-tail hypothesis is no solution to the problem. On the other hand Jefferies's views are much more complex⁷, but they are far more consistent, complete and, to my mind, compelling. □

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CANCER

A deadly inheritance

Bert Vogelstein

THE *p53* gene has led a short experimental life, but one filled with intrigue and irony. The latest twist is reported in two papers, one in *Science*¹ and one that appears on page 747 of this issue². Both show that inherited mutations in *p53* result in a predisposition to cancer.

Twelve years ago, *p53* protein was discovered through its association with the oncogene products of DNA tumour viruses^{3–5}. The gene encoding the protein was initially believed to be an oncogene because *p53* clones, including one obtained from normal mouse liver, could transform recipient cells^{6–8}. But several unexpected observations led to doubts about the precise role of *p53* in neoplasia. For example, it was shown that the *p53* clone from normal mouse liver had sustained a mutation during the cloning process; normal (wild-type) *p53* genes actually have no transforming ability. Furthermore, rearrangements and deletions of *p53* were observed in mouse erythroleukaemias and in a few human tumours^{9,10}. These alterations could have activated *p53* by removing growth-inhibiting portions of it (such as occurs in other oncogenes), but could also be interpreted as inactivating the gene product.

Two concurrent lines of investigation then brought *p53* into clearer focus. Many human tumours had been noted to have deletions of a small region of chromosome 17p which was subsequently found to include the *p53* gene. According to Knudson's hypothesis, chromosomal regions frequently lost in tumours are thought to harbour tumour-suppressor genes. So mutations in the *p53* gene remaining in tumours with chromosome 17p losses were sought — and found (see figure). The *p53* gene is the most commonly altered gene yet identified in human tumours, occurring in a large fraction (perhaps even half) of the total cancers in the United States and Britain.

Meanwhile, investigators continuing their studies of murine *p53* genes found that the wild-type form of the gene was not only inactive in transformation assays, but could actually inhibit oncogene-mediated transformation. Over the past few months, it has also been shown that

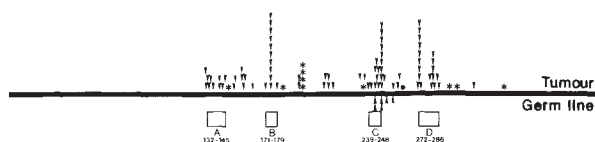
wild-type human *p53* genes, if expressed at adequate levels, can inhibit the growth of human cancers with endogenous *p53* mutations.

The current view is then as follows. At some point during tumorigenesis, wild-type *p53* activity becomes rate limiting for cellular proliferation due to its innate growth-suppressive ability. At that point, mutations in the gene can provide a growth advantage to the cell. Because *p53* protein apparently exists in cells in an oligomeric complex, mutant forms of it can exert a 'dominant-negative' effect, inactivating any wild-type protein that is complexed with it (and explaining the transforming activity of mutant *p53* genes). A subsequent deletion of chromosome 17 (usually through chromosome loss or mitotic recombination) can remove all residual wild-type *p53* protein from the cell, allowing complete escape from *p53* growth control.

Enter F. Li and J. Fraumeni, who through clever medical detective work discovered a group of families devastated by cancer. Autosomal dominant inheritance was consistent with the disease pattern, but linkage analysis to pinpoint the culprit gene was impossible: most of the families were small, affected members were usually dead, and it was often impossible to tell which of the live members were affected. Guesswork became the only alternative to identify the gene responsible. It has now been correctly guessed that the *p53* gene is inherited in mutant form in these kindreds^{1,2}.

This observation raises a host of questions. Why don't members of these families suffer from more tumours? Patients inheriting other suppressor gene mutations (neurofibromatosis, familial adenomatous polyposis, multiple endocrine neoplasia) often develop hundreds or thousands of neoplasms in the cell types at risk. Even patients with inherited mutations in the retinoblastoma gene often develop several retinoblastomas, though only a small number of retinal cells serve as potential tumour precursors. Given the diverse tumours occurring in Li-Fraumeni patients, it would seem that many human cell types are susceptible to

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Mutations in the human *p53* gene. The thick bar represents the 393 codons of the gene. Arrowheads represent missense mutations, asterisks denote nonsense mutations (due to deletions or point mutations) and closed circles represent splice-junction mutations. The mutations above the line have been documented in sporadic tumours (see ref. 11 for sources); those below the line have been shown to occur in the germ line of Li-Fraumeni patients^{1,2}.

the effect of inherited *p53* gene mutations; yet the median age of tumour development is over 30 years, and the median number of lifetime tumours is less than two. From the cell's (not the patient's) point of view, inherited *p53* mutations are incredibly weak tumour initiators.

This weakness is even more curious in light of the putative dominant-negative nature of the mutations described above. Perhaps some *p53* gene mutations are less dominant-negative than others; indeed, from recent studies in several laboratories it would seem that there are significant differences in the biological and biochemical activities of various mutant proteins. Interestingly, the mutant *p53* gene products in normal cells of Li-Fraumeni patients are apparently less stable (and therefore expressed at lower levels) than those previously analysed in sporadic tumours¹. But it is more likely that the low expression of the inherited mutant proteins reflects the cell type in which expression was assessed rather than the mutant type. And in fact some of the same mutant *p53* genes observed in Li-Fraumeni patients have previously been observed to encode high levels of stable proteins in the tumour cells of sporadic cancer patients. The striking positional clustering of the Li-Fraumeni mutations between codons 245 and 258 suggests however that the inherited mutations do indeed have some unique properties compared to most of those observed in tumours (see figure).

The new findings also illustrate an emerging concept in cancer gene research. Inherited *p53* gene mutations are clearly the initiating event in the tumorigenic process of Li-Fraumeni patients, occurring 10–30 years before the onset of malignancy. In many human tumours, however, *p53* gene mutations have been shown to represent late events in the neoplastic process, occurring 10–30 years after the initiation of neoplasia. There is thus a 30-year interval over which such mutations can exert an oncogenic effect. This emphasizes that it is not the time of occurrence of mutations, but rather their accumulation, that is most important in tumour development. In Li-Fraumeni patients, *p53* gene mutations occur first and other mutations follow. From the lower than expected incidence of cancer in

these families, it would seem that several additional mutations, not just loss of the wild-type *p53* gene, must occur before malignancy ensues. In the general population, these additional mutations occur first and *p53* gene mutations follow. The end result is identical, though, regardless of the order of the mutations.

Li-Fraumeni syndrome is rare — fewer than 100 families suffering from it have been identified. But what are the boundaries of the clinical features caused by mutations in *p53*? The clinical expression of many inherited diseases can vary widely depending on the mutation, the environment and the inheritance of other genes. It is clear from previous studies that *p53* gene mutations in most cancers are somatically acquired. It is possible, however, that a small percentage of such mutations (under 5 per cent) is inherited. This percentage may be higher for selected patient subsets, such as young women with breast cancer. If the inherited *p53* gene mutations are limited to the fewer than 0.001 per cent of breast cancer patients with classical Li-Fraumeni syndrome, this inheritance will have few practical implications for the general population. If, on the other hand, inherited mutations are found in a greater proportion (even as few as 1–5 per cent), screening for them will become a serious issue for public health policy.

The new results also have considerable historical implications. All of the recent work on *p53* gene mutations in humans can be directly traced to research on specialized and sometimes obscure experimental model systems. These older studies have now led to a key clue to the pathogenesis of many of the most common forms of human malignancy. In our increasingly goal-oriented research environment, the *p53* story, particularly its origins in basic research, may well be worth re-telling. □

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Aromatic air

LAST week Daedalus proposed synthesizing benzene in a magnetic field. Each ring molecule would then have a magnetic flux line threaded through it. Since the benzene ring acts as a room-temperature superconductor, through which flux lines cannot pass, the molecules would then be permanently linked to the flux lines like loose beads on a string.

Daedalus now muses that all benzene has been synthesized in a magnetic field anyway: that of the Earth. Any shipment of the compound must drag a little of the Earth's field with it. This of course explains the appalling navigational record of oil tankers and toxic-waste vessels: the benzene content of their cargo disrupts their magnetic compasses. It also suggests a novel global communication system. For benzene synthesized at one site and released as vapour would not diffuse randomly away. It would drift up the magnetic flux line leaving the Earth at that point, arch through space, and curve down again towards the 'conjugate point' where that flux line meets the other hemisphere. The benzene molecules threaded along the flux line would act as a sort of one-dimensional gas, and should therefore transmit sound. Being unable to spread out, this sound should travel along a flux line without attenuation.

DREADCO acousticians are already travelling to the magnetic conjugate points of major oil refineries and heavy-chemical installations. They reckon that benzene vapour released over the years will have saturated the connecting flux lines already, establishing a sonic channel. They will locate the exact conjugate point by listening for industrial noise echoing out of the empty sky; they will then shout back. The changing bandwidth and propagation time of the channel will give fascinating insights into the path, distributed benzene-loading, and high-altitude fluctuations of that particular flux line.

Daedalus is even inventing benzene molecule astronomy. Benzene released from a very northerly chemical works would ride the Earth's flux lines far into space. A shout or pulse of compressed benzene vapour launched up such a line should return an echo for each discontinuity it encountered. The high-altitude magnetosphere, the Earth's magnetotail stretching away from the Sun, the shock front where its field hits the solar wind, could all be studied from the relative comfort of some Northern tank farm or plastics factory. Some lines of force must even intersect the Moon, so that its changing distance could be measured by a sort of benzene-molecule sonar. The speed of sound is so low that it would take hours or days for the echoes to return. Distances established in this way would be correspondingly precise.

David Jones

Profiting from biodiversity

SIR—Many people see biodiversity as an expensive luxury which we either have a duty to pay for or simply cannot afford. A glance at almost any first-world dinner table, covered with food species from around the world, shows this is nonsense — biodiversity is an immense economic resource. Profitable and sustainable economic exploitation of biodiversity is perhaps the best way to ensure its maintenance. We recently attended a conference at Roros, central Norway in which Dan Janzen described a programme to turn Costa Rica into a country which profitably manages and exploits its biodiversity.

The first step is to take inventory. Janzen spends six months a year training 'parataxonomists' — former farmers, other rural workers and students — to provide the raw material for Costa Rica's National Biodiversity Institute. The institute itself, a private, non-profit making, public-service organization, integrates information on resources from the flora and fauna of the national parks which constitute more than a quarter of Costa Rica's land surface area with a view to exploiting it economically.

After training, the parataxonomists leave for designated patches of park to compile collections of local organisms. The collections are housed and handled at the institute by in-house curators. As the samples are identified to finer taxonomic levels, the curators call on expertise from abroad. The inventory already includes at least one potential moneyspinner. Janzen showed us a plant which, he claims, is rich in antibiotics. He refused to tell us the plant's natural location, as the intention is to develop its potential so that the economic benefits are channelled back into the preservation of Costa Rican biodiversity.

Janzen estimates that the contents of the major national parks should be catalogued within a decade: the first two classes of parataxonomists, comprising 33 students, are now working in the country's eight conservation areas. A local and regional infrastructure is now in place for the later stages of Costa Rica's biodiversity programme, which will include more fundamental research as well as commercial exploitation.

Who is paying for all this? The Costa Rican government, although an integral part of the enterprise, is not wealthy. Initially, a series of institutions, including private foundations, private donors and national governments, provided \$2.53 million for the first two years of operation. In the long term, one scheme is that initial capitalization of \$30 million would purchase \$120 million of Costa Rican international debt. The Costa Rican government would then buy the \$120

million by paying \$96 million to a biodiversity institute trust fund, which would pay 3% annual interest in local currency.

The National Biodiversity Institute represents a different attitude towards third-world biodiversity than that which characterizes the developed countries, where biodiversity tends to be viewed as a common heritage of mankind. This belief must be eliminated or else biodiversity will suffer the tragedy of the commons, with everyone trying to make a quick profit but unwilling to invest in sustaining the resource. If Costa Rica benefits economically from its biodiversity, then Costa Ricans will have an economic interest in the sustainable exploitation of their own natural resources. If successful, the institute will provide a model for similar schemes elsewhere.

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Ellipses and ellipsis

SIR—Klaczko and Bitner-Mathe in their Scientific Correspondence¹ fit ellipses to wing outlines of *Drosophila*. The fit is impressive and they suggest that ellipse shape can be used to quantify wing shape.

But the same ellipse can exactly fit outlines of very different shapes (Fig. 1). Such ambiguity arises when outlines are not closed, so that only part of the ellipse need fit. Klaczko and Bitner-Mathe digitize the wing tip and front margin (from A to D in Fig. 2). Although open, this portion is evidently sufficient to fit an ellipse representative of the entire, almost closed, wing periphery. But we may be interested in the open outline AD *per se* (developmentally, this and the hind margin belong to different compartments). In that case, to avoid equating dissimilar outlines requires two further parameters specifying which arc of the ellipse is used — for instance parameters θ and $\cos^{-1}(c/d)$ in Fig. 2 (top).

Unfortunately, although in combina-

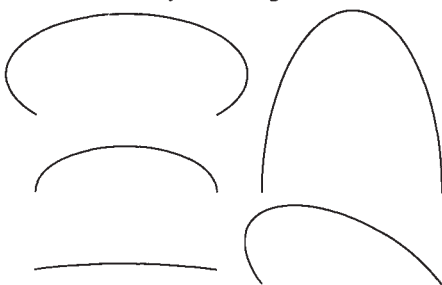


FIG. 1 Five arcs from the same-shaped ellipse.

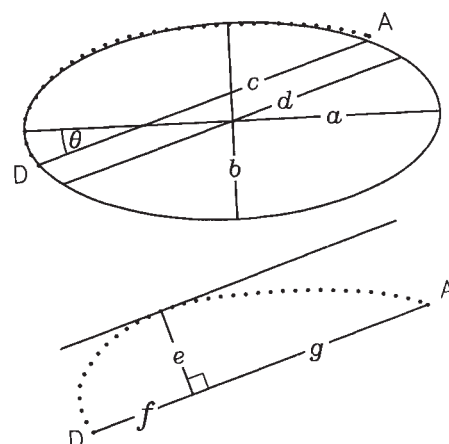


FIG. 2 Top, The dotted line AD represents the digitized wing outline, to which Klaczko and Bitner-Mathe fitted the ellipse. The line of length c cuts off the ellipse arc adjacent to the outline. Bottom, A simpler geometrical construction.

tion ellipse shape and these parameters specify fully the shape of an ellipse arc, each parameter remains difficult to interpret when the others are changing. Unless parameters can be chosen to match observed limits to variation, a change in outline recognizable as a single, simple distortion may affect all ellipse parameters in a confusing, nonlinear way. An ellipse arc geometrically intermediate between another two arcs need not have intermediate parameters; if parameters are intermediate, the arc need not appear geometrically intermediate (Fig. 3). Nor is the panacea to enter all the ellipse parameters in a multivariate analysis: we should

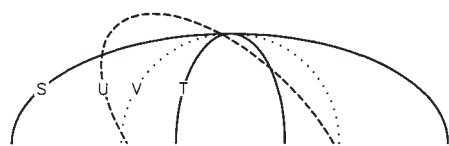


FIG. 3 Ellipses S, T and U share the same value of b/a and c/d , and U is intermediate in values of $\sqrt{ab}$ and θ . Ellipse V differs in b/a and so is not intermediate between S and T for this set of parameters.

not expect linear combinations to disentangle trigonometric inter-relationships.

A few-parameter summary of shape may be more comprehensible if based simply on relative distances between homologous landmarks and extremum points (see parameters $\log[e/(f+g)]$ and $\log[g/(f+g)]$ in the bottom part of Fig. 2). Alternatively, as wing venation provides a wealth of unambiguous landmarks, more advanced mathematical tools can be used to analyse shape variation².

Nevertheless, ellipses have been sensibly used to quantify biological shape³. Sampson has developed⁴ a fitting algorithm unaffected by the outline's orientation which generates confidence limits summarizing variation between outlines. The technique also allows ellipses to be constrained, for instance to lie parallel to

two landmarks. Each constraint discards information, but can make the remaining ellipse parameters easier to interpret.

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Crucifixion date

SIR—We have suggested^{1,2} that the most probable date for the crucifixion was on 3 April in AD 33, in part basing our claim on a lunar eclipse visible from Jerusalem on that evening. However, Clive Ruggles in *News and Views*³ discussed a paper by Schaefer⁴ claiming that this eclipse would not have been visible from Jerusalem. But there are several errors in Schaefer's work, so we do not think our conclusion needs to be revised.

We found that the eclipse of 3 April in AD 33 was visible from Jerusalem at moonrise: it rose with 20 per cent of its disk in the umbra and the remainder in the penumbra. The ancients, however, made no distinction between the umbral and penumbral shadows with the result that to the casual observer about 57 per cent of the Moon's disk would have been perceived as being 'in eclipse' at moonrise. Schaefer disputes this, maintaining that the rising Moon would first have become visible when only 1 per cent of its disk was still in the umbra and so the eclipse would have gone unnoticed.

The visibility of astronomical phenomena close to the horizon is determined principally by the amount of aerosol scattering in the line of sight. In estimating this, Schaefer takes the altitude of Jerusalem to be 450 m above mean sea level.

Scientific Correspondence

SCIENTIFIC Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words and 5 citations, and to contributions using simple language.

If new results are being described, priority is generally given to communications that do not describe work in which the author is involved. Authors of contributions of this nature should explain in a covering letter why theirs has a particular claim on *Nature's* space. Contributions may be sent to referees and, in the case of matters arising from material published in *Nature*, are sent to the author of that article for comment. A more detailed guide to authors is available from Washington or London. □

But the altitude of the old city is typically 775 m. Moreover, his correction factor for the effects of relative humidity is anomalously high. These two errors alone result in the amount of aerosol extinction at the horizon being overestimated by a factor of more than 700.

We would expect the equivalent of any astronomical phenomena seen from present-day Oxford to have been easily seen from ancient, pollution-free Jerusalem: the last three lunar eclipses visible from Oxford were all observed under less than ideal conditions at times when the Moon's altitude was considerably less than the value that Schaefer maintains is required for the Moon to be seen. Moreover, Schaefer's analysis denies the possibility of the simultaneous visibility of the Sun and eclipsed Moon as a result of atmospheric refraction — a phenomenon that has been known since the time of Hipparchus. Schaefer's analysis, based in part on a single observation of a lunar eclipse setting through the centre of the anthropogenic haze layer of Washington, DC, relies on recent measurements which are degraded by atmospheric pollution. We do not believe that the visibility conditions in ancient Jerusalem and modern-day Washington can be compared.

All calculations of ancient eclipses must take into account the cumulative effects of the inconstant rotation of the Earth due to effects such as tidal friction, for which we have adopted the results of Stephenson and Morrison, who analysed⁵ ancient astronomical observations. Schaefer estimates the required eclipse parameters by averaging several disparate eclipse calculations — among which at least one is defective and another is known to be incompatible with the well known eclipses of classical antiquity. After eliminating these two calculations from the set used by Schaefer we find excellent agreement with our own work (which Schaefer has misquoted).

At last umbral contact the Moon is still visibly in eclipse to the casual observer (Schaefer's analysis takes no account of this) and, as a result, the eclipse of 3 April in AD 33 would have been perceived by the general populace as continuing until about 51 min after moonrise. We therefore reaffirm that the partial lunar eclipse on that day would have been easily visible to the casual observer in Jerusalem. We have shown^{1,2} that this is the most probable date of the crucifixion and given textual evidence referring to a lunar eclipse following the crucifixion, Schaefer's paper⁴ does not provide grounds for doubting this conclusion.

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sion, which is based on the best available estimate of the clock error due to tidal friction⁵ and realistic values for the atmospheric extinction coefficient. We will provide a more detailed response to Schaefer's paper elsewhere.

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Vanishing authors

SIR—Cherry, in his *News and Views* article¹, gives a good account of the "case of vanishing neutrinos", but by referring only to Bahcall and Bethe for its interpretation, he creates a "case of vanishing authors". The significance of the third, or small-mass-difference, MSW solution was recognized long before the preliminary SAGE results were announced. J. M. Gelb and I were the first to describe its physical properties and to emphasize that it could yield a very small signal in gallium². E. W. Kolb, M. S. Turner and T. P. Walker independently arrived at the same conclusion³ and our numerical results were cast in analytical form by W. C. Haxton⁴ and by S. J. Parke⁵. Other authors refined and extended this work.

In August 1988, the Kamiokande II team announced its first measurement of 0.46 ± 0.15 for the fraction of solar neutrinos detected versus the standard solar model prediction. Gelb and I pointed out⁶ that the central value fell within the narrow range of values predicted by the third solution, but well outside the predictions of the high-mass solution. Unfortunately, the error was too large for us to draw a definite conclusion.

We did observe, however, that were the error cut in half and the central value left unchanged, then the high-mass solution could be eliminated and gallium could be used to choose between the other two. With the new results from Kamiokande II and SAGE, this is exactly what has happened.

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■ *Nature* severely restricts the number of citations in *News and Views* articles, which on this occasion accounts for the absence of reference to these papers in Dr Cherry's article.

A danger to science?

James E. Lovelock

The Rebirth of Nature. By Rupert Sheldrake. *Century*: 1990. Pp. 215. £14.99.

If Rupert Sheldrake had been a cosmologist, it would have been much easier to review his book; speculations about the universe range free and are judged by their entertainment value as well as by their scientific validity and use. But he is a biologist and at the present stage of perception biology seems vastly more complex than physics. My task is made difficult by more than just the complexity; the subject of the book falls within the discipline of evolutionary biology, a science that has long been in adversarial contest with religious fundamentalists over creation. Some evolutionary biologists have evolved to become like their opponents, dogmatic authoritarians, knowing and teaching the absolute truth; almost as if they were the Sunday School superintendents of a fundamentalist sect; wonderfully expert at sniffing out evil. Some of these zealots have already categorized my own work as dangerous and, aware of their influence, I felt uneasy when writing this review. This new inquisition will surely use anything favourable I say about someone they regard as a real heretic as evidence of my own delinquency.

Perhaps Lewis Wolpert was right to say "Sheldrake's ideas are just nonsense", but for others to call them dangerous, or a threat to science, goes too far. The theory of formative causation makes testable predictions, although nothing has yet been reported that would divert the mainstream of science. Even if it is nonsense, errors, in the golden days of science, were well regarded. As Vilfredo Pareto said of Kepler, we should "prize the fruitful error, full of seeds bursting with their own corrections." Recognizing this need for fruitful errors, I do not regard the book as dangerous. Ideas like morphogenetic fields or morphic resonance may not even be good guesses, but burning might give them that piquant smoked flavour of martyrdom. Entertaining and informative though it is, I do not think this book yet merits such an accolade. The question is, are the errors fruitful?

I think so, if only because they identify the real danger to science: the acceptance of a censorship of ideas and the isolation of science in a naive mechanical reductionism. In certain ways the scientific

establishment has grown to resemble Eastern Europe of a few years ago. Deviations from the materialistic creed are pilloried as threats to science, whereas patent nonsense, like cold fusion, is swallowed whole. Science is not threatened by the imaginative ideas of the Sheldrakes of the world, but by those who would censor them. Sheldrake is a threat, but only to the established positions of those

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Blue-tit trick — conventional learning or morphic resonance?

who teach and practice an authoritarian science. A healthy scientific community would accept or reject formative causation as the evidence appeared.

Some of us start a life of science motivated by a sense of wonder, but most now see science as an established career with the rewards of tenure and recognition for those who obey its inflexible rules. As a consequence, imagination is so disabled that naively we compare the exquisite complexity of living organisms, and their properties like intelligence, with the crudity of our trendy contraptions. Descartes, his disciples, and even opponents like Paley, thought of clockwork; steam engines with their control systems came later. I recall when the brain was compared to a telephone exchange, now of course it is said to be like a computer. What will it resemble next? This progression, where complex living entities that we do not understand are compared with the latest technological masterpiece, is human but absurd. Sheldrake is right to condemn

these uninspired mechanistic attempts to explain everything.

I believe that nature is objective and give thanks that we may never understand it. I know, from experience as an instrument designer and from trying to explain Gaia, that most scientists are blind to the circular logic of control theory. Even physiologists and systems engineers do not seem to realize that their view of organisms involves more than the simple reductionism of mainstream science, yet still is wholly objective. Understanding requires the top down view as much as the bottom up. The New Age antiscience that masquerades as holism is appealing because the lay public can sense the inadequacy of oversimplified reduction and are insulted by ingenuous attempts to blind them with science.

This is why Sheldrake's notions are popular with the Greens. Policy makers and scientists who need to understand the philosophy and motivations of the Greens would do well to read the book, which is a new window into biology; it covers topics not ordinarily discussed, like life and organisms. Generations of scientists have grown up unaware of a past that included fierce debates over vitalism and animism. These were as much a part of scientific history as was the phlogiston theory in physics. The book is partisan, but well written and rich in good quotations; it faithfully retreads the crooked path of the history of biology and of the metaphor of Mother Nature. Those who need ideas to stimulate their thoughts about the concept of Christian stewardship will be rewarded.

I disagree with Sheldrake's restatement of Gaia as a purposeful regulation of the material conditions of the Earth by the organisms; but he makes clear that this is his, not my, definition of Gaia. I wish that my opponents from the school of fundamentalist biology were as scrupulous. To Sheldrake, a biologist, the terms animate, alive and life imply purpose. To me, and to many engineers I suspect, the term alive also includes the concept of a system switched on and working, something very different from the same system switched off. Gaia is alive in this sense but in no way has foresight or purpose.

I enjoyed the book. Morphic fields and morphic resonance are fanciful ideas, even for a nonconformist like me, but not distasteful when taken with a pinch of salt. □

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Giant subjects

Nicholas Fraser

The Dinosauria. Edited by D.B. Weishampel, P. Dodson & H. Osmólska. *University of California Press: 1990. Pp. 733. \$85.*

NEXT year sees the 150th anniversary of the 'invention' of the dinosaurs by the eminent English anatomist and palaeontologist, Richard Owen. It was at the British Association meeting of 1841 held in Plymouth that Owen delivered an address

of the scientific validity of certain aspects of vertebrate palaeontological research. Although the last decade has witnessed a surge in the publication of academic papers on dinosaurs, the same period has seen an even greater production of popular books which usually concentrate on the visual impact of dinosaur restorations. Although some of these books are excellent and provide the reader with accurate and up-to-date information, too many have relied heavily upon speculation regarding dinosaur lifestyles. The publication of a book dealing with the dinosaurs in a rigorous scientific way is long overdue,

common-sense approach to speculation regarding the behaviour and physiology of dinosaurs. They do not dismiss the value of speculating on lifestyles of past life forms, but they do advise and emphasize caution. As a result there are no dogmatic claims; instead well balanced ideas are put forwards which are either backed by stable natural laws or are suggested by analogy with present-day land vertebrates.

Although there will inevitably be those who disagree with opinions expressed by the various authors (I admit that I would question some of the hypotheses put forward), the scientific merit of the book is to be highly commended, and all the hard data on dinosaurs are presented. This volume is a landmark in dinosaur research, and although I am sure that *The Dinosauria* would have met with the approval of Owen, he would surely have wondered why it took so long to produce a book of this calibre. I expect the volume to be the standard academic text on dinosaurs for a considerable length of time, but at the same time I hope we do not have to wait until the 300th celebration of Owen's major contribution to science before it is updated. □

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Mary Evans

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Stegosaurus — leaving a trail of fact mixed with fantasy.

on British fossil reptiles. Included within this paper were descriptions of *Hylaeosaurus*, *Iguanodon* and *Megalosaurus*, for which Owen introduced the term *Dinosauria*. It is perhaps appropriate that the British Association will again convene at Plymouth next year to celebrate the events of 150 years ago.

Since 1841 there has been a wealth of literature published on the dinosaurs and, particularly in recent years, a plethora of new forms have been described. The early papers resulting from dinosaur research were restricted to the scholarly journals and consequently they were not readily accessible to the enquiring mind of young children. Over the years, however, the incredible size of some of these extinct reptilian giants coupled with advances in technology have meant that the public, and in particular children, have been led along on a trail of fact mixed with fantasy. One of the results of this tenuous union between science and the entertainment world has been a somewhat sceptical view

and as a result *The Dinosauria* will be an invaluable addition to many scientific libraries. With the emphasis placed upon cladistic analysis it should help to restore faith in vertebrate palaeontology as a respectable science. I highly recommend it to anyone with more than just a passing interest in palaeontology, and dinosaurs in particular.

The vast majority of the book describes dinosaur taxonomy, and each group is dealt with in an authoritative manner by experts in their fields. Indeed the editors have done a magnificent job in bringing together the world's leading authorities on dinosaurs and producing the volume in a relatively short period of time (the project was first conceived in 1984). The comprehensive indices and the listing of the global distribution of dinosaur finds are indispensable. But in the light of the myths which often surround dinosaurs, I believe the two chapters on dinosaur palaeobiology to be especially important. Both Coombs and Farlow adopt a

New in paperback

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- Also from Longmans comes *Plant Physiology, Biochemistry and Molecular Biology* edited by David Denis and David Turpin, which provides an overview of recent advances particularly in the understanding of plant metabolism. Price is £19. □

Groundwork

Stephen Nortcliff

Soils of the British Isles. By B.W. Avery. CAB International: 1990. Pp. 463. £67.50, \$118.

It has always been with some embarrassment that we in the United Kingdom have had to explain to visiting soil scientists that there is no official classification for the soils of the United Kingdom, but rather separate systems for Scotland, England and Wales, and Northern Ireland. Of these systems, Avery the author of this text has had the greatest responsibility in developing the system for England and Wales. This system incorporates many of the currently accepted approaches to the classification of soils. The classification used in the book is not this scheme however, but a modified version based on his experiences with the system since its introduction. In many respects experience is the hallmark of this text. It is written at the end of a long and successful career in soil science and soil survey, and this clearly shows through in the authoritative and comprehensive style.

The nine chapters of the book are conveniently divided into two groups. The first group of three chapters is a broad introduction to the nature and pattern of soils and soil development and the manner in which attempts have been made to map the soil patterns and classify the soils, with a major emphasis on Britain and British

soils. The second group of six chapters deals in turn with one of the major soil groups identified as the highest level of the soil classification. These chapters are not solely lists of soil properties, but include brief introductory discussions of the relevant soil processes and environmental influences on soil development, together with the subdivisions of soil groups and subgroups. Although the soil descriptions may be widely available from other published sources, what gives the text a distinctiveness is the manner in which the soils are discussed and placed in a broad context. These discussions are supported with a number of black and white photographs of the landscapes within which the soils are found together with clear line drawings. There are relatively few photographs of soils; 16 small colour photographs are included in four pages of colour plates together with a small number of within text black and white photographs. This limited number of photographic illustrations is the only negative point I would make about the text, and even this is offset to a large degree by the excellent soil descriptions.

In conclusion, this is an excellent text which will serve for many years as a reference base for anyone involved in the study of soils in the British Isles. It is comprehensively and clearly written and a most welcome addition to the soil science literature. □

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All tied up

John D. S. Jones

The Geometry and Physics of Knots. By M. Atiyah. Cambridge University Press: 1990. Pp. 78. £6.95, \$14.95.

ONE of the arts in doing mathematics is to find the right point of view — from which things become clear — the elegant solution. Taken to extremes this becomes the purist's search for the ultimate elegance and style and it usually obscures essential issues, but in the right measure this search is one of the most important things in mathematics — in its simplest terms the most elegant explanation is usually the most convincing one. Mathematics is full of deep interactions between seemingly different questions and these links do not usually become clear until one has the right point of view.

Michael Atiyah's book is a brief and rapid introduction to the incredible mixture of mathematical ideas and techniques in the work of Vaughan Jones and Edward Witten relating knots and quantum field theory, and it is a superb example of how the right point of view can be so revealing. This material is at the forefront of the current, deep and exciting interaction between geometry and physics and, in particular, of the way quantum field theory appears to involve very significant features of geometry and topology in three and four dimensions. Many of the insights which led to this renaissance are due to Atiyah, so it is particularly interesting to read him expressing some of his ideas on the subject. It is also quite extraordinary to realize that the theory of knots should provide one of the focal points in the present interaction between geometry and physics.

Knot theory is the branch of mathematics that studies simple closed curves in space. Simple means the curve does not cross itself and closed means the curve ends where it started — imagine taking a piece of string, tying a knot in it and then sewing the two ends together so you cannot slip the knot off. You will immediately get the right impression of the complexity of the subject if you experiment. In 1984, Vaughan Jones discovered a new technique in knot theory which led to a flurry of activity and many new insights. In its simplest possible terms, Jones' method is to draw a diagram of the knot on a piece of paper, indicating under-crossings and over-crossings in the usual way, and then to read off, from the pattern of crossings, a polynomial. This polynomial is an invariant of the knot, which means that two different diagrams of the same knot yield the same polynomial. Most important of all, it is extremely good at distinguishing between different knots.



Igloos, built from blocks of wind-packed snow cut with long knives and stacked up in a spiral fashion, can last for several weeks in the polar winter. This picture is taken from *North Pole, South Pole* by Bernard Stonehouse, which examines life at the poles and polar issues. Published by Prion, price is £17.95. □

Even though it can be expressed in very simple terms, this is a very sophisticated piece of mathematics. Jones' discovery was generated by finding a different point of view on a piece of work he had done on a seemingly unrelated problem.

One conceptual problem raised by Jones' work is that although knot theory is intrinsically three-dimensional, after all a knot is a curve in three-dimensional space, his technique is to draw a two-dimensional picture and then to read off the invariant. Of course this does not affect the usefulness nor the importance of his work. The problem of finding a conceptual three-dimensional interpretation was solved by Edward Witten using techniques from quantum field theory, an area of physics which is seemingly quite unrelated to knot theory.

Atiyah concentrates on explaining some of the geometrical ideas behind Witten's approach to the Jones poly-

nomials and how this fits in with other aspects of Witten's most recent work in quantum field theory and its relationship with geometry and topology. The exposition is admirably lucid and elegant and I cannot imagine a better introduction, for graduate students and the more experienced, for mathematicians and physicists, to the large amount of high-powered mathematics required. The book is an expanded version of a series of lectures, and, as in his lectures, Atiyah achieves such a degree of clarity by emphasizing ideas and motivation rather than details — the reader who would like to see a detailed account will have to look elsewhere but will be well prepared to appreciate the more technical aspects of the theory. As a source of insight this book is marvellous. □

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Homo turmoil

Michael Day

The Evolution of *Homo erectus*. By G. Philip Rightmire. Cambridge University Press: 1990. Pp. 260. £32.50, \$44.50.

Of the three stages we know of the evolution of man (the australopithecine apemen, *Homo erectus* the first true men, and early *Homo sapiens* our own species) *Homo erectus* of the Middle Pleistocene would have seemed the most clearly understood and the most taxonomically stable of them all a relatively few years ago — not any more. Important new finds as well as new ways of thinking about hominid taxonomy have thrown this 'species' into the same turmoil as all of the others.

The first find that was attributed to this taxon was that of Java Man, found at the turn of the century by the Dutchman Eugene Dubois: this was followed by the finds of Peking Man in China by Davidson Black in the 1920s and the further finds of the great Ralph von Koenigswald in the 1930s and 1940s. This group of material formed the basis for Louis Leakey's theory that man arose as an Asian species rather than one from Africa. The 1960s found Louis Leakey ahead in this debate when his recovery of a *Homo erectus* calvaria from Bed II at Olduvai and of *Homo habilis* from the Lower Pleistocene deposits of Bed I in Olduvai Gorge seemed to shift the cradle of humanity firmly to Africa. The discoveries of Richard Leakey from the region of Lake Turkana, north Kenya, of both australopithecines and hominines in the 1970s and 1980s added to this view of human origins. This phase of discovery was crowned by the find of the 'Turkana Boy', a young

skeleton that is attributed to *Homo erectus* and is almost complete and believed to be 1.6 million years old.

Europe and north Africa have also seen the discovery of new material — such as that from Bilzingsleben in Germany and Petralona in Greece — to add to the well-known Mauer jaw and Ternifine remains and attributed by some to *Homo erectus*.

In short, what was a relatively clear picture of Middle Pleistocene hominine evolution has become turbid and debate has centred around all of the following questions. Does *Homo erectus* exist as a true species or should it be sunk into *Homo sapiens*? Is it a palaeospecies that exists in true form as a segment of the line that emerges from *Homo habilis* and gave rise to *Homo sapiens*? Is *Homo erectus* an extinct form that had no part to play in the evolution of *Homo sapiens*? Is there a good example of *Homo erectus* in the European fossil record? Finally, are the Asian forms of *Homo erectus* so far removed from the evolution of *Homo sapiens* in Africa to cast doubt on the existence of *Homo erectus* in Africa at all? *The Evolution of Homo erectus* is the personal testimony of Philip Rightmire, and some of these questions, but not all, are answered by him on the basis of comparative anatomical studies that have extended over many years and the

examination of almost all of the original material.

On this basis there is no doubt whatsoever that the work is authoritative and contains extended anatomical descriptions of the skulls and jaws: the postcranial remains and teeth, perhaps mercifully, command less detailed attention. It finally becomes clear that Rightmire believes that *Homo erectus* is indeed a species "spread from Africa to Asia by the onset of the Middle Pleistocene" and again "*Homo erectus* is a real palaeospecies rather than an arbitrary grade or stage in the evolution of a lineage". The question of the existence of this taxon in Europe is left open but the direct but gradual transformation of *Homo erectus* into *Homo sapiens* is not supported from this review of the evidence.

The style of writing is in keeping with the man, calm, assured and competent, but the text is a little dull. On the other hand, the best operative surgeon that I ever watched was the duller — nothing ever went wrong and there was no drama. *The Evolution of Homo erectus* is not a textbook for undergraduate students, not a monograph or a handbook, but a useful volume on a topical debate that professionals will consult frequently. □

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Fatherly love? — An adult male gorilla (left) looks closely at one of his offspring, seeming the image of the doting father. *The Mountain Gorilla* by Boyd Norton is a delightful portrayal of these animals and the myths that surround them. Published by Swan Hill Press, price is £16.95. □

The origin of large local uplift in extensional regions

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Large localized uplift is commonly observed in continental regions undergoing extension. These observations can be modelled by planar, high-angle normal faulting of an elastic upper crust overlying an inviscid lower crust. Isostasy provides the necessary driving force. The model quantifies the role of flexural rigidity, density variations in the crust, and erosion and deposition of sediment.

MANY authors have noted that uplift of several kilometres can occur in extensional regions. The uplift is usually local in extent, extending 10–20 kilometres perpendicular to the strike of the structures and many tens of kilometres along strike. Evidence for uplift, possibly as much as 15 km, comes from local stratigraphic relations, metamorphic mineral assemblages, and fluid-inclusion studies in rocks that must have been generated in the middle or lower crust. The origin of such large localized uplift is a subject of lively debate, centering on the geometry of the normal faults and the role of isostasy and crustal flexural rigidity.

Here we show that the observations may be explained quantitatively by simple planar high-angle faulting of an elastic upper crust overlying an inviscid lower crust that is subject to isostatic forces. The model is consistent with observed gravity profiles and earthquake source mechanisms^{1–3}, and with the observation of crustal shortening in the upper parts of footwalls. We illustrate the model with a number of examples of structures from the Basin and Range province in the United States.

The model

The approach to modelling these structures follows from that used by King *et al.*¹ and Stein *et al.*². There the mechanical behaviour of the crust was modelled as an elastic layer overlying a fluid. The vertical component of motion at the surface was calculated using the thick-plate solution described by Rundle⁴. It was shown that the main features of the geological structures associated with some dip-slip faults could be explained if the fault was assumed to be planar through an elastic-brittle layer dipping at 45° or greater and if the effects of loading due to erosion and sedimentation were considered. The most surprising feature of the model was the need to reduce the effective elastic thickness from the 10–15 km depth of the seismogenic zone to between 2 and 4 km.

Here we adopt a different computational approach using a boundary-element system modified, as described below, from that of Crouch and Starfield⁵. This allows us to extend the earlier work and calculate both vertical and horizontal displacements and strains for two-dimensional models. We use a version of the boundary-element technique that uses constant relative displacement elements. This approach is very effective. A minimum of computational steps allow many models to be run on a small computer and because the approach is easy to understand avoiding numerical errors is straightforward.

The technique consists of introducing a series of dislocation elements into an elastic medium. The location and amplitude of slip on the elements is then adjusted such that stresses and displacements at boundaries and interfaces reasonably approxi-

mate the real problem being examined.

The two-media problem we examine can be expressed as a solution of the following equations

$$\begin{aligned} b_s^i &= \sum_{j=1}^N C_{ss}^{ij} D_s^j + \sum_{j=1}^N C_{sn}^{ij} D_n^j \\ b_n^i &= \sum_{j=1}^N C_{ns}^{ij} D_s^j + \sum_{j=1}^N C_{nn}^{ij} D_n^j \end{aligned} \quad (1)$$

where $i = 1-N$. The b_x^i are displacements ($b_x^i = u_x^i$) of or stresses ($b_x^i = \sigma_x^i$) on the i th boundary element. For normal components $x = n$ and for shear $x = s$. The D_x^j are normal ($x = n$) or shear ($x = s$) displacements between the faces of the j th boundary element, and the C_{xy}^{ij} are the influence coefficients between the i th and j th elements. Subscripts indicate normal (x or $y = n$) or shear (x or $y = s$) components.

The first N_1 elements are in region 1 and the remainder $N_2 = N - N_1$ are in region 2. These elements may either represent a free part of the boundary of region 1 or region 2 or a segment on the interface between the two regions. If the i th element is an interface element in region 1 then a corresponding interface element i^* must exist in region 2.

Four conditions must be satisfied at an interface and hence relate any pair of interface elements. These may be conveniently expressed as two on the region 1 element and two on the region 2 element.

Two stress conditions are imposed on the region 1 element

$$\begin{aligned} \sigma_s^{i(1)} - \sigma_s^{i^*(2)} &= 0 \\ \sigma_n^{i(1)} - \sigma_n^{i^*(2)} &= 0 \end{aligned} \quad (2)$$

where $\sigma_x^{(1)}$ corresponds to an interface stress in region 1 and $\sigma_x^{(2)}$ corresponds to an interface stress in region 2. The stress conditions are satisfied in equation (1) if

$$\begin{aligned} b_s^i &= \sigma_s^{i(1)} - \sigma_s^{i^*(2)} = 0 \\ b_n^i &= \sigma_n^{i(1)} - \sigma_n^{i^*(2)} = 0 \\ C_{ss}^{ij} &= A_{ss}^{ij} \quad j \leq N_1 \\ C_{ss}^{ij} &= -A_{ss}^{i^*j(2)} \quad N_1 + 1 \leq j \leq N \end{aligned} \quad (3)$$

The $A^{(1)}$ are influence coefficients relating stress to displacement for region 1 and $A^{(2)}$ are similar coefficients for region 2. C_{sn}^{ij} , C_{ns}^{ij} and C_{nn}^{ij} are defined similarly. On the region 2 element, the following displacement conditions are imposed

$$\begin{aligned} u_s^{i(2)} + u_s^{i^*(1)} &= 0 \\ u_n^{i(2)} + u_n^{i^*(1)} &= 0 \end{aligned} \quad (4)$$

which are satisfied in equation (1) if

$$\begin{aligned} b_s^i &= u_s^{i(2)} + u_s^{i^*(1)} = 0 \\ b_n^i &= u_n^{i(2)} + u_n^{i^*(1)} = 0 \\ C_{ss}^{ij} &= B_{ss}^{ij(1)} \quad j \leq N_1 \\ C_{ss}^{ij} &= B_{ss}^{ij(2)} \quad N_1 + 1 \leq j \leq N \end{aligned} \quad (5)$$

The $B^{(1)}$ are influence coefficients relating displacement to displacement in region 1 and $B^{(2)}$ are similar coefficients for region 2.

With the appropriate values for element stress, displacements

and influence coefficients, the $2N$ equations can be solved for the $2N$ values of D by the usual methods. Once the D are known it is straightforward to calculate displacements anywhere in region 1 or 2 for which valid expressions can be written in terms of D . The procedure for setting up influence coefficients in an elastic media is straightforward and is described in Crouch and Starfield⁵. The behaviour of a horizontal gravitating interface between an elastic medium (region 1) and an inviscid fluid medium (region 2), for which the pressure change with depth is H , can be expressed if the region 2 influence coefficients take the following values

$$A_{nn}^{ij(2)} = \begin{cases} H^i & \text{if } i=j \\ 0 & \text{if } i \neq j \end{cases} \quad (6)$$

$$B_{nn}^{ij(2)} = \begin{cases} 1 & \text{if } i=j \\ 0 & \text{if } i \neq j \end{cases}$$

All other $A^{(2)}$ and $B^{(2)}$ are zero.

In our models the x_1 axis is vertical and positive downwards and the x_3 axis is horizontal. Two horizontal gravitating interfaces are defined. One represents the Earth's surface ($x_1 = 0$ km) and the second, the base of the brittle layer ($x_1 = 12$ km). At $x_3 = -60$ km the horizontal displacement along a vertical boundary is fixed ($u_3 = 0$) and the shear stress is set to zero ($\sigma_{13} = 0$). At $x_3 = 48$ km both shear and normal stresses are set to zero ($\sigma_{33} = \sigma_{13} = 0$) at a second vertical boundary. These conditions produce a stress-free floating block 'tethered' at -60 km. The length of the block is sufficient that details of the way in which the boundary conditions are imposed do not affect the deformation in the region where faulting is introduced.

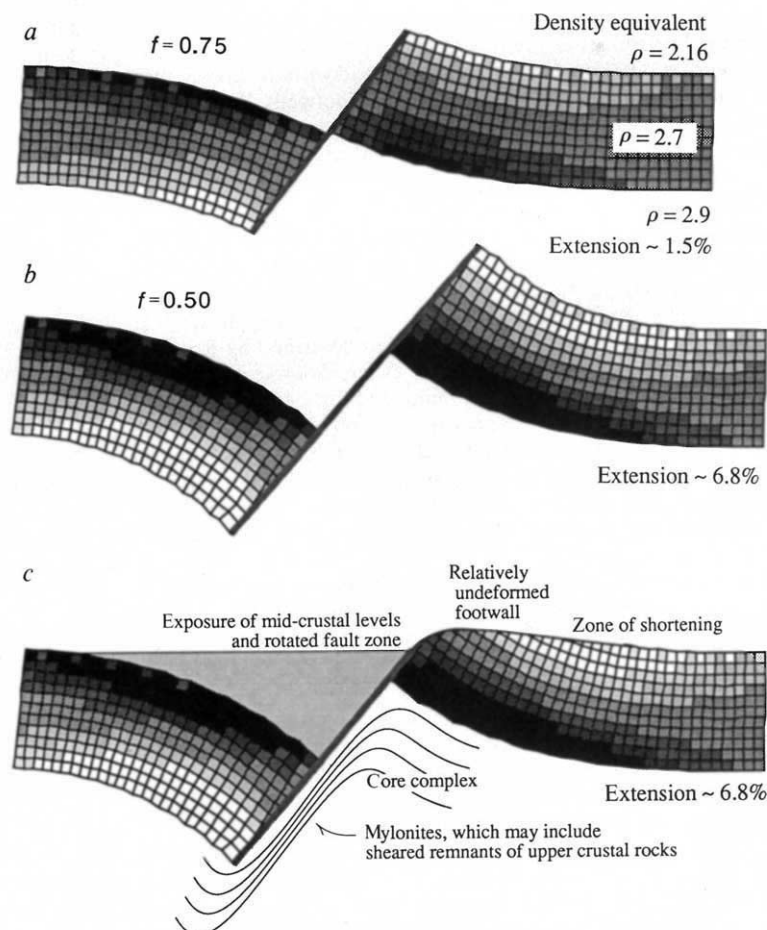
The driving force to deform the block is derived in the following manner. In the Earth we may suppose that the upper crust is able to sustain a certain magnitude of deviatoric stress over a time of millions of years. We further assume that over the

same time repeated motion on major faults in the crust is appropriately modelled by assuming that the fault is effectively of zero strength. The nature of this assumption is discussed by Rundle⁶. Because we wish to examine the stresses and strains due to the fault displacement only (rather than these combined with any initial state of stress) we effectively turn the situation around in the numerical model by allowing the initial state of stress to be zero (hence the boundary conditions above) and by driving displacement with a shear stress τ_0 applied to the fault. Thus, specifying the right-hand vertical boundary condition in our model as $\sigma_{33} = 0$ is not equivalent to assuming that σ_{33} is actually zero in the Earth. The effective σ_{33} in the real world determines our choice of shear stress across the fault. In a gravitating block with free boundaries the vertical stress at depth h is $\sigma_{11} = h\rho g$. The resolved shear stress on a plane dipping at an angle θ is given by $\tau = (\sigma_{11} - \sigma_{33}) \sin \theta \cos \theta$, and its average by $\tau_0 \approx \tau/2$. Assuming a density of upper crustal material of $2,700 \text{ kg m}^{-3}$, a fault dip of 60° gives $\tau \approx 140(1-f)$ MPa, where $f = \sigma_{33}/\sigma_{11}$. To model a fault of zero long-term strength, we therefore apply $\tau_0 \approx 70(1-f)$ MPa.

The choice of f is open to debate. If $f = 0$, this implies that the crust can reach the verge of going into tension. Even with displacement boundary conditions this is unlikely to occur. Other have proposed values between $1/3$ and 1 , based on near-surface measurements or on models much simpler than ours (for example, ref. 7). We therefore choose $f = 0.5$, which we regard as a likely maximum stress difference. For comparison, we also show the result for $f = 0.75$.

At the base of the elastic layer the fluid has a density of $\rho_3 = 2,900 \text{ kg m}^{-3}$ and in the absence of erosion and sedimentation, the density above the elastic layer would be $\rho_1 = 0.0$. A convenient way to represent the effects of erosion and sedimentation is to substitute a medium of significant density for the air. It would be most straightforward to choose a medium with a

FIG. 1 Representative boundary-element models shaded with dilatational strain at a 0.02 contour interval. The grid units represent square kilometres and are for scale only. Dark areas represent extension (areal increase), light areas contraction (areal decrease). The strains are shaded from 0.5 km to a depth of 11.5 km and represent those due only to the motion across the fault and not due to any preexisting condition or body forces. These plot limits (0.5–11.5 km) avoid the large local strains associated with the singularities at the end of the boundary elements. Effective elastic thickness is ~ 3 km assuming a crustal Young's modulus of 40 GPa and a Poisson ratio of 0.25. Density below the layer is $2,900 \text{ kg m}^{-3}$. We assume 80% erosion and sedimentation, which corresponds to assuming a density above the top surface of $2,160 \text{ kg m}^{-3}$. The fault and distant shear boundaries are single elements whereas the upper and lower boundaries are in 1.5-km segments in the central region, increasing to 6-km segments outside the region shown. The vertical boundary at -60 km is fixed, and at $+48$ km $\sigma_3 = 0$. *a* and *b* show a fault model driven by a shear stress that corresponds to a ratio of vertical to horizontal stress in the crust of 0.75 and 0.5, respectively. *c*, A modification of *b* illustrates the effect of erosion and sedimentation and is annotated by commonly observed features. The same calculations were carried out for element lengths of 3 and 6 km. The resulting displacements were the same to within a few per cent, but the strain patterns became ragged when fewer, longer elements were used. In both models, shortening occurs in the upper part of the footwall, and extension in the lower part. Uplift of the footwall is significant; in particular, *b* and *c* show the ease with which mid-crustal levels can be exposed at the surface with relatively minor extension.



density related to the proportion of underlying material removed by erosion or added by sedimentation. For example, choosing a medium of density $2,160 \text{ kg m}^{-3}$ is mechanically equivalent to removing 80% of the uplifted mass by erosion and replacing 80% of the mass removed by subsidence through sedimentation. Were this the case, the value for H below the elastic layer would be $g\rho_3$ and that above would be $g\rho_1$. Net forces applied to the plate that resists flexure are proportional to $(\rho_1 - \rho_3)g$, and forces proportional to $(\rho_1 + \rho_3)g$ are applied across the plate creating strains within the plate. Such a model does not allow for the reduction of modulus E to E_{eff} (where $E/E_{\text{eff}} = 60$), introduced to allow the elastic layer to possess a long-term effective elastic thickness of $\sim 3 \text{ km}$. In the absence of a correction, unreasonably large strains appear in the layer. A number of corrections are possible. The flexure is largely determined by the term $(\rho_3 - \rho_1)g$, which must remain the same. On the other hand, $(\rho_1 + \rho_3)g$ must be reduced by E_{eff}/E . This may be achieved if we replace ρ_1 by $\rho_1 E_{\text{eff}}/E$ and ρ_2 by $(\rho_2 - \rho_1)(1 - (E_{\text{eff}}/E))$.

The foregoing provides one approach to applying boundary conditions to the layer, but others are possible. For example, if displacements are infinitesimal such that points do not displace with respect to a fixed axis system, then H at the base becomes $(\rho_3 - \rho_2)g$ and at the surface $(\rho_2 - \rho_1)g$.

Within reasonable limits, details of the way these boundary conditions are applied do not affect the final result, provided that the net restoring force remains proportional to $(\rho_3 - \rho_1)g$. They may be chosen according to what is perceived to be the physical processes that cause effects such as the long-term loss of strength of the elastic layer. Unfortunately, the insensitivity to details of these boundary conditions also has the consequence that data of the sort we describe cannot be used to determine more detail about the behaviour of the plate.

Some representative models are shown in Fig. 1. In Fig. 1a–c the fault dips at $\sim 60^\circ$ and cuts through a 12-km-thick layer of density $2,700 \text{ kg m}^{-3}$. Although the elastic layer is 12 km thick we use a Young's modulus such that its equivalent flexural rigidity (10^{20} N m) is the same as an elastic layer $\sim 3 \text{ km}$ thick, assuming a crustal modulus of $\sim 40 \text{ GPa}$. Our models support the previous result^{1,2} that only models with an effective elastic layer 2–4 km thick yield sensible geological structures. Models with greater elastic thickness produce structures wider than those observed, and those with a smaller elastic thickness produce structures that are too narrow.

Figure 1a and b shows the deformation for $f = 0.75$ and $f = 0.5$, respectively. The results can be interpreted in several ways. For example, if $f = 0.5$ is considered to be the maximum stress difference, then the deformation shown in Fig. 1b is the maximum that can be achieved by gravity alone. If this is the case, then the result for $f = 0.75$ (Fig. 1a) may be interpreted as 50% of the maximum deformation, or as the maximum deformation if the fault is capable of sustaining half of the available shear stress. The significance of the model, however, is separate from these interpretations: large local uplift of the footwall driven only by gravity is an unavoidable result of a realistic dynamic model.

Figure 1c is the same as Fig. 1b but shows the effects of erosion and sedimentation and is annotated with commonly observed features. In particular, it shows the relatively undeformed but rotated footwall, the zone of shortening in the upper part of the footwall, exposure of mid-crustal level rocks, and the inferred development of mylonites in the inviscid lower crust.

The figures are shaded to indicate strain changes due to fault motion. Areal strain ($\epsilon_{11} + \epsilon_{33}$) is plotted with lighter shades representing contraction and darker shades dilation. The slight checkerboard effect at the upper and lower boundaries are associated with singularities at the ends of the elements. (See also the caption to Fig. 1.) At the surface, dilation occurs in the hanging wall and contraction occurs in the footwall. Field observations of gentle to close folds and conjugate strike-slip faults in the footwall reflect the contractional strains produced

in the upper part of the model footwall. At the base of the elastic layer the conditions are reversed with contraction in the hanging wall and extension in the footwall. We regard the dilational strains at the base of the footwall as significant, and we suggest elsewhere (ref. 8 and M.E. and G.K., manuscript in preparation) that the implied volume increase may provide a mechanism to tap locally available partial melt, which eventually ascends to produce the commonly observed flank volcanism.

Field examples of finite structures

Many examples of large localized uplift occur in the Basin and Range province, two of which are shown in Fig. 2. Examples share the characteristics that uplift and tilting is confined to a relatively narrow (~ 10 – 20 km) zone parallel to the associated high-angle normal fault, and minor shortening occurs in the upper part of the footwall. It is also commonly observed that volcanism and shallow intrusives occur preferentially in the footwall.

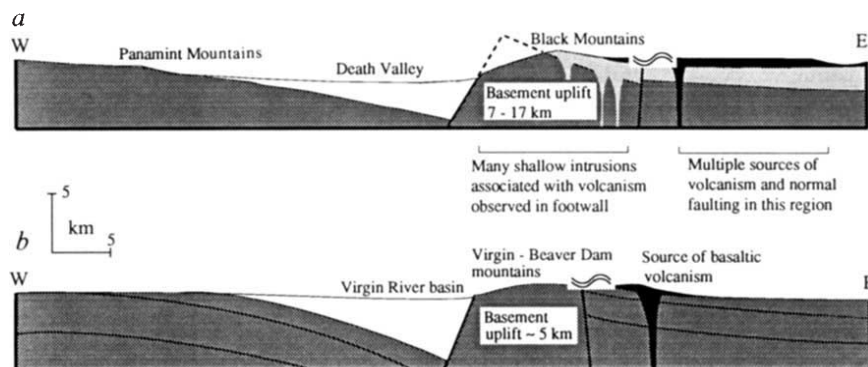
Central Death Valley (Fig. 2a) is an asymmetrical east-tilted graben, bounded to the east by an active high-angle, range-front normal fault that bounds the western side of the Black Mountains⁹. The present range-front has been active since at least 10 Myr, although most of the uplift has probably occurred in the past 6 Myr (ref. 8). Gravity measurements over the valley floor⁹ and seismic reflection¹⁰ and refraction information¹¹ suggest that the basin fill is 3–5 km thick.

Significant local uplift of between 7 and 17 km and the eastward tilt of the Black Mountain block, confined to a relatively narrow 10–20 km perpendicular to the main fault, is evidenced by exposure of middle-crust rocks near the range-front fault and lower-grade, stratigraphically higher rocks to the east^{9,11–14}, by aluminium geobarometry analysis of a local mid-Tertiary intrusion¹⁵ and by the geometry of tilted extrusive rocks¹¹. Total displacement across the main fault zone is therefore 10–22 km. Pliocene basalts exposed near their source in the footwall are gently folded along axes parallel to the main normal fault.

The active Virgin-Beaver Dam fault system (Fig. 2b) marks the eastern edge of the transition zone between the southern Great Basin and the Colorado Plateau. The system was initiated during the middle Miocene and is responsible for $\sim 6 \text{ km}$ of subsidence in the hanging wall and a minimum of 5 km of uplift in the footwall (R. E. Anderson, personal communication). The footwall exposes Precambrian basement in an eastward tilted block and preserves an older part of the normal fault (the Castle Cliff detachment) now stranded at a low angle on top of the basement culmination. The Palaeozoic and Mesozoic cover sequence is gently to closely folded within, and parallel to, the footwall of the Beaver Dam section and is shortened by conjugate strike-slip faults in the Virgin Mountain section. Significant Tertiary basalts of local origin are also observed in the footwall. The age of the folds is not precisely known, although various arguments suggest that they are associated with the development of the main normal-fault system¹⁶.

Further examples from the Basin and Range province include the Shoshone and Cortez ranges in northern Nevada^{17,18}, the Humboldt and Stillwater ranges of north central Nevada^{17,19}, the Wassuk range of central Nevada^{20,21}, and the Wasatch range in Utah. Each of these ranges, except the Humboldt, is associated with a large active range-bounding normal-fault system, which was initiated in the middle Miocene and which is associated with volcanic rocks and (or) shallow intrusions in the footwall. Folds of Pliocene units are observed in the footwalls of the Stillwater²² and Wassuk ranges²⁰. The adjacent basins are about 3–4 km thick and displacement across the normal faults is at least 4–5 km^{17,19}. The Humboldt fault system seems to be less developed (1–2 km displacement) and is probably a younger system. The presence of relatively young volcanic rocks (late Pliocene or possibly Pleistocene) confined to the footwall of the Humboldt range supports the supposed young age of the fault. The Wasatch range and its associated basin is a particularly

FIG. 2 Examples of structures from the Basin and Range province having large localized uplift along high-angle planar faults. Dark shading indicates pre-Tertiary basement. Light shading indicates middle Miocene volcanic rocks or intrusions associated with the early stages of faulting. White indicates basin-fill sedimentary units, black indicates basalts. *a*, Death Valley, data from refs 9 and 11. Uplift of footwall may be up to 17 km based on geobarometry studies¹⁵, although the regional stratigraphic column probably limits displacement to <10 km. Source of Miocene volcanics lies within the footwall of the main fault. *b*, Virgin-Beaver Dam Mountains (cross-section supplied by R. E. Anderson, personal communication).



striking example of the potential importance of erosion and sedimentation. Stratigraphic arguments²³ and fluid-inclusion studies²⁴ suggest that the Wasatch range has a vertical offset of ~11 km, and seismic reflection and gravity suggest that the basin is only ~4 km deep²⁵. This type of structure may be modelled by allowing the system to be open to mass change (see also refs 1, 2), such that the rate of erosion is relatively high (promoting uplift), and the rate of sedimentation is relatively low (inhibiting subsidence). Seismic reflection, gravity models and earthquake seismology suggest that the faults described here, and in general for the Basin and Range province, are high-angle and planar^{26,27}.

The models shown in Fig. 1 are generally consistent with these examples. In particular, they produce the high uplift of individual ranges while retaining the relatively narrow dimension of each basin and range, and they correctly predict shortening in the elbow of the footwall.

Discussion

Other investigators have addressed some of the observations described here, and have recognized that a successful model must incorporate elements of the model we propose^{28,29}. The numerical modelling illustrates that a simple isostatic model incorporating only high-angle faults explains the observations very effectively.

Localized and rapid uplift in extensional regions has been attributed to the isostatic response of a previously suppressed crustal root broken by high-angle faulting³⁰. Our analysis avoids the difficulty of assuming that the crust can maintain isostatically unbalanced roots for extended periods of time before the faulting occurs (such as in ref. 31) and of assuming that each highly displaced and elevated range was underlain by a preexisting root. Our model provides a means to produce large uplift and displacements from an elastic upper crust that was originally in isostatic equilibrium.

A controversial topic in Basin and Range tectonics is the role of low-angle faults in extension of the upper crust³². Our model shows that the appropriate structural and topographic cross-sections can be produced by planar high-angle faulting of the upper crust. We do not dispute the field evidence for low-angle faulting and suggest that minor low-angle normal faulting may be produced along the higher sections of the main fault as it is rotated with the footwall. Eventually the high part of the fault will be abandoned (for example, ref. 29) and will continue to be rotated and uplifted in the footwall. The turtlebacks of central Death Valley³³ and the Castle Cliff detachment of the Beaver Dam Mountains represent examples of this abandonment process.

Low-angle faults will also be produced by imbrication of the footwall (commonly observed in extensional regions), a process in which slices of the footwall are transferred into the hanging wall and subsequently rotated to a lower angle. This process, as well as imbrication of the hanging wall, also has the effect of juxtaposing severely attenuated upper-plate rocks against highly sheared and mylonitized rocks of the lower plate. This is a fundamental characteristic of metamorphic core complexes.

The finite structure of Fig. 1*b* is likely to be the maximum deformation possible given a gravitational driving force, a reasonable density structure and flexural strength, and the minimum plausible ratio of horizontal to vertical stress. Large extension of the continental crust requires the development of many such structures, giving rise to a distributed basin and range structure. This distinguishes our model from the rolling footwall hinge model^{29,30}, which requires that substantially more uplift should readily occur.

Controversy centres around estimates of crustal extension in the southern Basin and Range. We have shown that significant uplift of the footwall can occur with only minor horizontal extension (Fig. 1). Wernicke *et al.*³⁴, however, suggest that crustal extension at this latitude is ~300% (~250 km), based on palinspastic reconstructions of displaced stratigraphic and structural features. It is possible that much of this displacement, which is in any case disputed³⁵, may be accomplished by a combination of strike-slip faulting (see, for example, refs 36, 37) and large volumes of intrusives, as observed in the Death Valley region of California.

Conclusions

Our numerical model resolves a number of difficulties with previous ideas about the origin of large localized uplift in extensional regions and quantitatively examines factors such as flexural rigidity, density differences, and erosion and deposition of sediment. Others have introduced these ideas but have only considered them in isolation or in qualitative terms. A striking feature of the models is that the modelled flexural rigidity of the elastic upper crust is consistent with results found by other techniques^{1,2,38}, and the choice of high-angle planar normal faults is independently justified by results from earthquake seismology^{26,27}. □

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The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily

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The *let-23* gene is required for induction of the *Caenorhabditis elegans* vulva. It is shown that *let-23* encodes a putative tyrosine kinase of the epidermal growth factor receptor subfamily. Thus, *let-23* might encode the receptor for the inductive signal required for vulval development. Because *let-23* acts upstream of *let-60 ras* in the vulval determination pathway, the identification of the *let-23* product provides support for a link *in vivo* between tyrosine kinase growth factor receptors and *ras* proteins in a pathway of cell-type determination.

DURING *Caenorhabditis elegans* vulval induction, the combined action of several intercellular signals specifies the precise pattern of fates of the six precursor cells (VPCs)^{1–4}. The main determinant of this pattern is an inductive signal from the gonadal anchor cell that stimulates the nearest three VPCs to proliferate and generate vulval tissue; the remaining three cells generate nonspecialized hypodermis^{5–7}. A 'lateral signal' acts between VPCs to ensure the proper pattern of cell fates³. Genes necessary for vulval induction have been identified by 'Vulvaless' mutations that prevent induction^{4,8–13,14}. One such gene, *let-60*, encodes a *ras* protein¹⁵. Other genes, identified by 'multivulva' mutations, prevent vulval differentiation in the absence of inductive signal^{3,4,8–11,16}. One such gene, *lin-15*, seems to act in cells other than the anchor cell or VPCs¹⁷, suggesting the presence of a negatively acting signal from a third tissue such as the surrounding hypodermal syncytium hyp7.

The *let-23* gene, originally identified by a larval lethal mutation¹⁸, has a pivotal role in *C. elegans* vulval determination: loss of *let-23* function leads to none of the VPCs having vulval fates, but some mutations in *let-23* result in too many VPCs having vulval fates (refs 10 and 11; and R.V.A. and P.W.S., manuscript in preparation). Here we report that *let-23* encodes a protein of the epidermal growth factor (EGF) receptor tyrosine kinase subfamily. On the basis of its molecular structure and previous genetic data, we propose that *let-23* normally functions to receive an inductive signal, presumably from the anchor cell, and acts to specify cell type. With previous data indicating that *let-23* acts upstream of the *let-60 ras* gene in the vulval determi-

nation pathway^{13,15}, our results provide evidence for a link *in vivo* between a growth factor receptor and *ras*.

Genetic and physical maps of *let-23* region

We localized *let-23* on the *C. elegans* physical map^{19,20} by identifying physical markers that map genetically to the left and right of the gene (Fig. 1). Three-factor mapping positioned the previously identified restriction fragment length polymorphism (RFLP) *Tc5A* roughly 80 ± 70 kilobases (kb) to the left of *let-23* (see Fig. 1 legend). We found the left breakpoint of the deficiency *mnDf67*, cloned the junction fragment, and 'jumped' to the right breakpoint, which then defined a right-most boundary for *let-23* (see Fig. 1 legend). Thus, the 200-kb region of DNA between *Tc5A* and the *mnDf67* right breakpoint contains the *let-23* gene and centres around the cosmid T08E2. Three-factor mapping data with the *Tc5A* RFLP suggested that *let-23* is in the cosmid T08E2 (Fig. 1), which contains a tyrosine kinase gene (see below). Efforts to detect allele-specific RFLPs in this 200-kb region were thwarted by the presence of extensive areas of repetitive DNA (data not shown).

Isolation of a tyrosine kinase gene near *let-23*

In parallel, a wild-type *C. elegans* genomic library was screened with a 0.75-kb *EcoRI-PvuII* fragment of the oncogene *v-ros*²¹ encoding the tyrosine kinase domain. Six classes of hybridizing clones were obtained. The hybridizing regions in these clones were partially sequenced. A coding sequence for a putative tyrosine kinase catalytic domain was found in each of the clones, and the sequences were found to all be different (M.K. and Y.O., unpublished observations). The clones were placed on the *C. elegans* physical map. One clone, NGros213–13.3, was mapped to linkage group II near *let-23* and is completely contained in the cosmid T08E2. The cloned gene was designated *kin-7*. A restriction map of the *kin-7* gene is shown in Fig. 2, as are various constructs referred to later.

The kinase gene rescues *let-23* defects

To determine whether *let-23* and *kin-7* are the same gene, we performed germline transformation experiments (Fig. 2). Cosmid and plasmid DNA were injected into the germ line of a balanced *let-23* lethal strain to test for rescue of the *let-23* lethal phenotype. Because the *let-23* allele used also gives rise to defects in the vulva and in fertility, we could assay rescue of these phenotypes as well.

The overlapping cosmids T08E2 and ZK1052, both of which

contain *kin-7*, rescue mutants from *let-23* defects, whereas the neighbouring but nonoverlapping cosmid W07A12 does not (data not shown; see Fig. 1 for cosmid locations). The subclone pK7-13.8, which contains the entire kinase gene and several kilobases on either side, rescues mutants from *let-23* defects (Fig. 2). But 5' and 3' deletion derivatives of pK7-13.8 (subclones pK7-5.5 and pK7- Δ TK respectively) that both truncate within the gene and share about 1 kb of overlap, fail to rescue (Fig. 2). Therefore, the rescuing activity results from the kinase gene and not another gene on either the 5' or 3' end of pK7-13.8. Furthermore, failure of both pK7-5.5 and pK7- Δ TK to rescue is not due to any abnormal properties of these plasmids (such as accidental point mutations), because we could achieve rescue by coinjecting the two plasmids (Fig. 2). Homologous recombination *in vivo* would allow these two plasmids to generate an intact *kin-7* gene. The plasmid pK7-11.5 does not rescue *let-23* mutants from lethality (Fig. 2). As this construct retains the entire *kin-7* coding sequence and about 1 kb of upstream sequence, sequences essential for rescue lie more than 1 kb upstream of the initiator methionine codon. The plasmid NGros213-13.3 (Fig. 2), which deletes the 3' end of pK7-13.8, including about 600 base pairs (bp) of C-terminal complementary DNA sequence, but retains the kinase domain and 200 bp downstream, rescues *let-23* lethality, suggesting, together with the pK7- Δ TK results described above, that the kinase domain is important for rescue. Our conclusion that *let-23* is *kin-7* was confirmed by the localization and sequencing of a point mutation associated with a *let-23* null allele in the region corresponding to the kinase domain (see below).

Wild-type mRNA and cDNA Clones

A 4.9-kb strong band and a 3.5-kb faint band were detected on

northern blot hybridizations (Fig. 3) of poly(A)⁺ RNA prepared from a mixed population of wild-type N2 strain probed with a 2.0-kb nonrepetitive *let-23* genomic fragment (Fig. 2). The 4.9-kb major band corresponds to the *let-23* cDNA (see below). The faint band could represent a cleavage product of the 4.9-kb messenger RNA, a minor form of the *let-23* transcript or a transcript of another kinase gene with similarity to *let-23*.

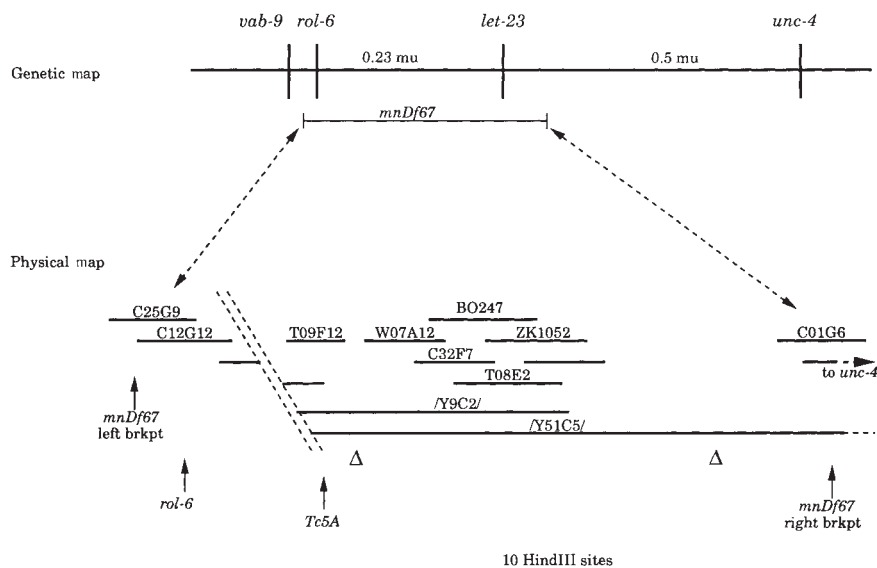
Roughly 7×10^5 plaques of a cDNA library from mRNA of a mixed population of *C. elegans* were screened with the 2.0-kb *let-23* genomic fragment. Only one clone, Cros331, was obtained. This clone had a 2-kb cDNA for the C-terminal half of *let-23*. To obtain the entire coding sequence, 13 oligonucleotide primers were prepared, based on the genomic sequence and its predicted exon-intron structure. Poly(A)⁺ RNAs were reverse-transcribed using oligo 1 and oligo 5 as the primers (Fig. 4), and amplified by PCR using oligonucleotide primers. The amplified sequences were cloned into plasmids. Because all the primer pairs lay across at least one intron, the possibility of amplifying genomic DNA could be excluded.

The assembled *let-23* cDNA sequence contains a single open reading frame that encodes 1,323 amino acids with ATG (position 80) as the initiation codon (Fig. 4). There is a poly(A) tail at the 3' end of the cDNA. This cDNA seems to lack a few hundred bases corresponding to the 5' end of the mRNA, because it is 4.3-kb long without poly(A) and the mRNA size was estimated to be 4.9 kb. The entire coding sequence, however, is covered as there is an in-frame stop codon (position 22) upstream of the putative initiator methionine codon.

The *let-23* allele *sy5*, which behaves genetically like a null mutation and is a homozygous larval lethal (R.V.A. and P.W.S., manuscript in preparation), has a point mutation in the region corresponding to the conserved kinase subdomain IX (ref. 22),

FIG. 1 Genetic and physical maps of the *let-23* region. A continuous stretch of cosmid DNA extends from the cosmid containing *rol-6* to the cosmid T09F12 (data from the *C. elegans* physical map^{19,20} and A. Coulson, personal communication). Between T09F12 and *unc-4* are numerous gaps in cosmid DNA that are spanned by yeast artificial chromosome clones (YACs)²⁰. T08E2, cosmid T08E2; Δ , location in gaps in cosmid stretches; /Y9C2/, YAC Y9C2; brkpt, break-point; arrows point to location of cosmid that contains stated molecular markers; mu, map units.

METHODS. (1) Tc5A three-factor mapping: We mapped the previously identified Bergerac/Bristol polymorphism, Tc5A (J. Park, personal communication), relative to *rol-6* and the *let-23* recessive lethal allele *sy15* (R.V.A. and P.W.S., manuscript in preparation; for details of mapping with Bergerac-specific polymorphisms, see ref. 44). We constructed the strain *rol-6(e187)let-23(sy15)/vab-9(e1744)* Tc5A from the strain *vab-9(e1744)* Tc5A *rol-1(e91)* (provided by A. Papp; *rol-1* maps to the right of *unc-4*). The *rol-6 let-23* chromosome is from the Bristol strain and lacks the Bergerac-specific polymorphism Tc5A. The *vab-9* chromosome has DNA sequences between *vab-9* and *unc-4* derived from the Bergerac strain and contains the Tc5A polymorphism (a novel 3.2-kb *EcoRI* fragment that appears when a genomic Southern blot is probed with the T09F12 subclone pAH1). We selected 20 Roller (Rol) non-lethal recombinants, made the recombinant *rol-6* chromosome homozygous, and isolated genomic DNA from these recombinants using standard protocols⁴⁵. We digested the DNAs with *EcoRI*, ran them on a 1% Tris-borate-EDTA polyacrylamide gel, blotted the gel onto a nylon membrane, and probed the blot with plasmid pAH1. Sixteen recombinants displayed the Bergerac pattern, indicating that *let-23* is to the right of Tc5A and is 1/4 (4/16) the distance from *rol-6* to Tc5A. Because the interval between *rol-6* and Tc5A contains 113 *HindIII* sites (the metric used in the *C. elegans* physical map data base), our three-factor data suggests *let-23* is 28 ± 25 *HindIII* sites (assuming the average *C. elegans* G-C content of 36%, one *HindIII* site occurs about every 3,000 base pairs). To locate *let-23* on the molecular map, we estimated the size of the gap in the cosmid map to the right of the cosmid T09F12.



We sized the YAC Y9C2 that spans this gap on a pulsed field gel⁴⁶ as 180 kb, and, subtracting for the amount of cosmid DNA covered by this YAC, estimated the gap to be about 10 kb or 3 *HindIII* sites. The next gap to the right was similarly estimated to be 24 *HindIII* sites by sizing the YAC Y51C5. Our best estimate of 28 *HindIII* sites from Tc5A therefore positioned *let-23* in the cosmid T08E2. (2) *mnDf67* breakpoint mapping: To define a marker on the right side of *let-23*, we mapped the right breakpoint of the deficiency *mnDf67* (ref. 47) that lies to the right of the *let-23* gene. The left breakpoint of this deficiency lies between two cloned markers, *rol-6* (ref. 48) and a candidate for *vab-9* (J. Kramer, personal communication). DNA from a cosmid, C25G9, detected a novel 10-kb *SalI* fragment in DNA isolated from animals heterozygous for *mnDf67*. This fragment was cloned from a size-selected λ phage library of *mnDf67* DNA and was used to probe the *C. elegans* physical map. In addition to hybridizing to the cosmid C25G9, the *mnDf67* junction fragment hybridized to the cosmid C01G6 which lies to the right of Tc5A and left of *unc-4*.

which changes the TGG codon encoding Trp 1,078 to an opal codon (TGA) (Fig. 4). This mutation would result in a truncated protein lacking some of kinase subdomain IX and all C-terminal sequences. That this allele results in a null phenotype is consistent with studies of tyrosine kinases that suggest that the C terminus of the catalytic domain resides close to, but downstream of, the conserved arginine in subdomain XI (ref. 22). Furthermore, such a truncation lacks rescuing activity: injection of plasmid pK7-13.8 digested by *SalI* (*SalI* cuts once in genomic sequence; see Fig. 2) to give a kinase gene deleted for kinase subdomains X and XI and all sequences 3' of this, fails to rescue (data not shown).

let-23 is a member of the EGF receptor subfamily

The predicted product of the *let-23* gene has several remarkable primary structures: two hydrophobic stretches, a putative

tyrosine kinase domain, and two cysteine-rich motifs. The general architecture (Fig. 5) is the same as that of human EGF receptor²³, *Drosophila* EGF receptor²⁴ (abbreviated DER), and the *Xiphophorus* *Tu* locus²⁵ (not shown).

One of the two hydrophobic stretches follows the initiator methionine and a basic residue (Arg) and seems to be a leader sequence for insertion into the membrane²⁶. The other hydrophobic stretch, which lies in the middle, is most probably a transmembrane domain because its 23 amino acids have a hydrophobicity index²⁷ of 2.6 and make it long enough to span a membrane; also, this sequence is flanked by charged residues.

Figure 6a shows the alignment of the kinase domains of the *let-23* product and representative tyrosine kinases of five subfamilies²². The *let-23* domain shows highest similarity (44.0% identity) to the human EGF receptor. It also shares 40.6% identity with DER, 31.6% with the human cellular *src* product,

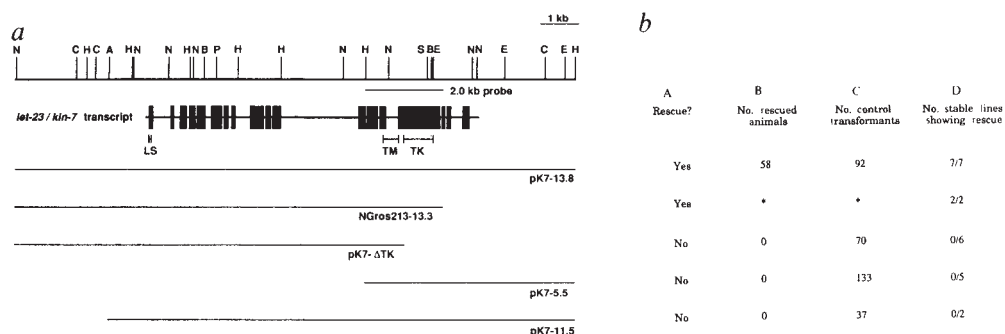


FIG. 2 Genomic organization and germline transformation. *a*, Restriction map of the *kin-7* genomic region is shown at the top. Location of the *let-23/kin-7* transcript (cDNA) is shown in the next row down, with amino-acid-encoding regions shown as solid boxes. The 5' end of the transcript is not determined. Locations of the genomic regions in the subclones used are depicted below. LS, leader sequence; TM, transmembrane domain (interrupted by an intron); TK, tyrosine kinase catalytic domain. Restriction enzyme sites: N, *NsiI*; C, *SacI*; H, *HindIII*; A, *Apal*; B, *BamHI*; P, *PstI*; S, *SalI*; E, *EcoRI*. *b*, Results of germ-line transformation experiments for each plasmid. The bottom row are the results for coinjection of pK7-ΔTK and pK7-5.5 (see text and below). Column A: 'Yes', given plasmid rescues the lethality associated with a *let-23* null allele; 'No', plasmid does not rescue. Column B: number of animals rescued in the first generation (F1) after injection. Column C: number of animals showing expression of a coinserted dominant marker and serves as a control for successful injection. Column D: number of stably transformed lines displaying rescue relative to the total number of stably transformed lines tested. Concentrations of plasmid DNAs ($\mu\text{g } \mu\text{l}^{-1}$): 50, 35, 18, 50 and 80 + 40 for pK7-13.8, pK7-ΔTK, pK7-5.5, pK7-11.5 and pK7-ΔTK + pK7-5.5, respectively. All plasmids were used at nearly uniform molarity except for pK7-ΔTK and pK7-5.5, which were coinjected at about twice the relative molarity of the others. Our results are insensitive to *let-23* dosage: we have separately injected pK7-ΔTK and pK7-5.5 at 50 $\mu\text{g } \mu\text{l}^{-1}$ and pK7-13.8 at 10 $\mu\text{g } \mu\text{l}^{-1}$ and 250 $\mu\text{g } \mu\text{l}^{-1}$ with no change in results. We have also coinjected pK7-ΔTK and pK7-5.5 at 40 and 20 $\mu\text{g } \mu\text{l}^{-1}$ respectively and found F1 rescue (two Rol Unc animals). Asterisk denotes that rescued F1 animals were found, but total numbers of rescued and control animals were not determined.

METHODS. Genomic clones: 3.9×10^4 plaques of an EMBL4 library constructed from an *MboI* partial digest of *C. elegans* N2 genomic DNA (gift from C. Link) were screened with the 0.75-kb *EcoRI*-*PvuII* fragment of *p-ros-1*, a plasmid clone (gift from K. Shimizu) of the *v-ros* oncogene²¹ at a low-stringency hybridization. Nylon membranes (Biodyne A) were prehybridized at 42 °C for 3 h in 20% formamide, $6 \times \text{SSC}$, $5 \times \text{Denhardt's}$ solution, 0.2% SDS, $500 \mu\text{g } \text{ml}^{-1}$ of salmon sperm DNA. About $0.5 \mu\text{g}$ ^{32}P -labelled probe DNA (specific activity $> 1 \times 10^8$ c.p.m. μg^{-1}) was added and hybridization was performed under the same condition for about 24 h. The filters were washed at 42 °C for 3 h with $3 \times \text{SSC}/0.1\%$ SDS, and exposed to an X-ray film (Kodak X-OMAT AR) for 24–40 h with an intensifying screen. The 11.5-kb *EcoRI* insert DNA of NGros113, a phage clone carrying *kin-7* gene, and 5.5-kb *HindIII* fragment of a cosmid clone B0247 (Fig. 1) (gift from J. Sulston and A. Coulson) were subcloned into plasmid pBluescript SK(+) to make NGros213-13.3 and pK7-5.5, respectively. These two plasmids were the sources of *kin-7* gene clones for later experiments. Germ-line transformation: We injected plasmids/clones into the strain *let-23(mn23) unc-4(e120)/mnC1(dpy-10(e128) unc-52(e444))*. The *let-23* allele *mn23* (ref. 17) results in 100% penetrant larval lethality and behaves like a genetic null (R.V.A. and P.W.S., manuscript in preparation). The balancer *mnC1* inhibits recombination in the region around *let-23* (ref. 18). We injected into the distal arm of the gonad syncytium of 1- to 2-day-old adult hermaphrodites according to the method of C. Mello, V. Ambros, J. Kramer and D. Stinchcomb (manuscript in preparation). The plasmid DNA of interest was coinjected with a plasmid (pRF4, courtesy of J. Kramer) bearing a dominant *rol-6* mutation (*rol-6(d)*; concentration of pRF4, 50 $\mu\text{g } \mu\text{l}^{-1}$ for all experiments). Expression of pRF4 in an

otherwise wild-type worm results in a Roller (Rol) phenotype. Appearance of F1 Rol progeny after injection therefore indicates successful injection and expression of injected DNA. Previous experiments have shown that homologous, coinjected plasmid DNAs form large linear arrays containing both DNAs (C. Mello, personal communication). All plasmids used in our experiments contain large regions with homology to pRF4 owing to the presence of vector sequences. About 10% of F1 Rol animals could stably transmit the arrays to some of their progeny, thereby resulting in some F2 Rol animals (and therefore F3 and so on). If a plasmid rescues *let-23* lethality, then these stable lines will segregate viable *Unc-4* (abbreviated *Unc*) animals, which if not suppressed would die as young larvae owing to the presence of the linked *let-23(mn23)* mutation. The number of stable lines showing rescue out of the total number of stable lines generated for a given experiment is displayed in column D. In all cases where we had stable lines but no rescue, we verified the presence of the *unc-4* chromosome by mating Rol animals with *rol-6(e187) unc-4(e120)/mnC1* males and looking for *Unc* progeny. This confirms that the failure to see *Unc* animals was not due to a rare recombination event that eliminated the *unc-4* marker. Rescued *Unc* animals are often Rol, indicating that the plasmid and *rol-6(d)* DNA are coexpressed; sometimes they are not. Nonetheless, in the next generation (if fertile) some Rol animals always segregate, suggesting that the *rol-6(d)* DNA is present in these non-Rol *Unc* animals but that the expressivity of the *rol-6(d)* phenotype is weaker than *let-23* rescue. In extensive negative control experiments with DNA from other chromosomes, stable Rol lines never segregate *Unc* or Rol *Unc* worms, consistent with lack of *let-23* rescuing activity. Rescued *Unc* animals are often sterile, indicating that the lethality, but not the sterility, associated with loss of *let-23* function is rescued (R.V.A. and P.W.S., manuscript in preparation). This partial rescue could be due to requirements for higher dosage for overcoming *let-23* sterility and/or mosaicism of the array in a given animal: the array may be lost in lineages where it is required to rescue sterility. Fertile Rol *Unc* and *Unc* animals are often capable of laying eggs, indicating rescue of the *let-23* vulval defect. The presence of F1 Rol *Unc* and *Unc* animals from injected mothers is an even more sensitive assay than segregation of stable Rol lines for *let-23* rescuing activity. In this assay, *rol-6(d)* DNA is a control for successful injection and the presence or absence of *Unc* animals indicates rescue or lack of rescue of *let-23* lethality. The number of *Unc* animals (Rol and not Rol) is given in column B; the number of Rol (not *Unc*) animals is given in column C). There is perfect correlation between the two assays: DNA which results in F1 *Unc* and Rol *Unc* animals also gives rise to stable Rol lines which segregate *Unc* and Rol *Unc* animals, and DNA which does not result in F1 *Unc* and Rol *Unc* animals gives rise to stable Rol lines which do not segregate *Unc* and Rol *Unc* animals. Rescue in the pK7-ΔTK and pK7-5.5 coinjection experiments (at both concentrations) is less efficient than rescue by pK7-13.8, but this is not unreasonable given that rescue requires *in vivo* recombination between the two plasmids to create a functional gene. Although the two plasmids overlap by about 1 kb within the kinase gene, they also share about 3 kb of identical vector sequence; homologous recombination in this region would not reconstruct a functional kinase. Three of the five F1 *Unc* animals in the coinjection experiment shown were also Rol, indicating the presence and expressivity of injected DNA. Lastly, we have also rescued the lethal allele *mn216* (ref. 47) and the *Vulvaless* allele *sy97* (R.V.A. and P.W.S., manuscript in preparation) (data not shown).

Localization of the *sy5* mutation: We localized the *sy5* point mutation by hydroxylamine mismatch detection⁵² (R. Barstead, personal communication). We PCR-amplified 750 bp of the kinase domain from homozygous wild-type and *sy5* genomic DNA (isolated from dead larvae), hybridized the mutant and wild-type DNAs, subjected the hybrids to hydroxylamine and piperidine reactions, and gel-electrophoresed the reaction. A 170-bp cleavage fragment was detected in three independent PCR reactions, and the PCR product was sequenced from a gel slice without subcloning (D. Nickerson, personal communication). The location of the *sy5* mutation is consistent with the 170 bp fragment generated.

32.0% with the human cellular *abl* product, 28.6% with the human insulin receptor, and 32.0% with the mouse PDGF receptor. The *let-23* domain has 36 of 39 consensus amino-acid residues thought to be involved in specific aspects of kinase activity²². For example, the invariant lysine in subdomain II seems to be directly involved in the phosphotransfer reaction²⁸. The *let-23* putative kinase is likely to be tyrosine-specific as subdomains VI (DLATRN in the single-letter amino-acid code) and VIII (AIKWLAIE) are more similar to the tyrosine kinase consensus (DLAARN or DLRAAN in subdomain VI and PI/VK/RWT/MAPE in VIII) than to the serine/threonine kinase consensus (PLKPEN in VI and GT/SXXY/FAPE in VIII)²². But the *let-23* domain has no tyrosine residues within 20 residues upstream of AIE in subdomain VIII, whereas many tyrosine kinases have an autophosphorylation site in this region²⁹.

The alignment of the extracellular domain of the *let-23* product with those of human and *Drosophila* EGF receptors is shown in Fig. 6b with respect to cysteine residues and the amino-acid spacings between them. There are two cysteine-rich motifs (see also Fig. 5) where cysteine residues occupy identical positions with interspersions of other amino acids. The amino-acid identity between the *let-23* product and DER or the human EGF receptor is 33.2% or 28.8% in motif I, and 33.9% or 35.2% in motif II-1. Cysteine-rich motif II-2 is found in the *let-23* product and DER (22.0% identity) but not in the human EGF receptor. There is also limited similarity in the presumed ligand-binding domain located between cysteine-rich motifs I and II-1 (26.6% identity with DER and 28.7% with the human EGF receptor). We conclude that *let-23* is a *C. elegans* member of the EGF receptor subfamily.

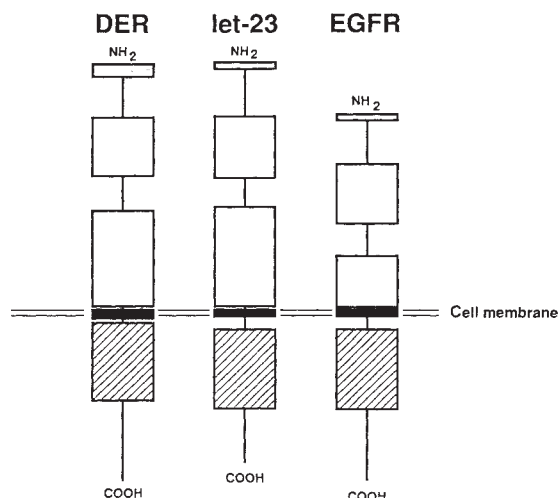


FIG. 5 Comparison of schematic structures of *let-23* with human and *Drosophila* EGF Receptors. Cysteine-rich regions, open boxes; transmembrane domains, solid boxes; tyrosine kinase catalytic domains, hatched boxes; leader sequences, dotted boxes. There are three strongly homologous regions (see text and Fig. 6 for details).

let-23 may encode the inductive-signal receptor

As mentioned above, the *let-23* gene is necessary for the induction of VPCs by the gonadal anchor cell and is thought to have a pivotal role in vulval determination. Furthermore, our analysis suggests that *let-23* does not act in the anchor cell, because a

FIG. 6 Comparison of amino-acid sequences (single-letter code) of *let-23* and other tyrosine kinases. *a*, Alignment of the amino-acid sequence of the putative tyrosine kinase catalytic domain of *let-23* and those of six other tyrosine kinases: EGFR, human EGF receptor^{24,53}; SRC, human cellular homologue of the oncogene product from Rous sarcoma virus⁵⁴; Abl, human cellular homologue of the oncogene product from Abelson murine leukaemia virus⁵⁵; INSR, human insulin receptor⁵⁶; PDGFR, mouse platelet-derived growth factor receptor⁵⁷. Asterisks, invariant residues; +, invariant except *let-23*; dots, identical to *let-23*; dashes, space for the alignment. *b*, Alignment of *let-23*, DER and EGFR extracellular cysteines. Numbers indicate the number of amino acids between cysteines. Regions with identical amino-acid spacing between cysteines among two or three of the kinases are boxed. MB, membrane.

<i>a</i>	Subdomain	I	II	III	IV
<i>let-23</i>	(883)	LQTKLDKKLGAGAGTGVFAGIYYPKRAKNVKIPVAIKVFQTDQSQSTD--EMLEEATNMFRLL-RHDNLLKIIIGFCMHDDGL			
EGFR	(686)	TEF.KI.V.S.....YK.LWI.EGE.....ELREAT.PKANK.I.D.YV.ASV-DNPHVCRLL.I.LT-STV			
DER	(857)	AELRKGGV.M.....R.YK.VWV.EGEN.....ELLKSTGAESSE.F.RE.YI.ASE-E.V.....LLAV.S-SQM			
SRC	(265)	ESLR.EV...Q.C.E.WM.TWNG-----TTR...TLKPGTM--SPEAF.Q.VV.KK...EK.VQLYAVVSE-EPI			
Abl	(240)	TDITMKH...G.QY.E.YE.VWKK-----YSLT.V.TLKE.TM--EVE.F.K.AV.KEI-K.P..VQLL.V.TREPPF			
INSR	(982)	EKIT.LRE..Q.S.M.YE.NARDIKGEAETR.V.TVNEA.LRERI.F.N.SV.KGF-TCHHVVRLL.VVSKGQPT			
PDGFR	(566)	D.LVIGRT.S.....Q.VEATAHGLSHSQATMK.V.MLKSTARSSKQALMS.LKI.SH.GP.L.VVNL.L.A.TKGGPI			

<i>b</i>	Subdomain	V	VI	VII
<i>let-23</i>		KIVTIYRPLGNLQNLKLKHEN-----LGAREQVLYCYQIASGMQYLEQRVVRDLATRNVLVKKFNHVEITDFGLS		
EGFR		QLI.QLM.F.C.LDYVRE..D-----I.SQYLLNW.V...K.N...DR.L.....A.....TPQ..K.....A		
DER		MLI.QLM..C.LDYVRNRNDK-----I.SKALLNWST..K.S...EK.L.....A.....RLL-AG.DH.....A		
SRC		Y...E.MSK.S.LD..KGETGKY-----RLPQL.DMAA.....A.V.RMNY.....RAA.I..GENLVCKVA....A		
Abl		Y.I.EFMTY...LDY.RECNRQE-----VN.VVLLYMAT..S.A.E...KNFI.....A.C.GENHL.KVA....A		
INSR		LV.MELMAH.D.KSY.RSLRPEA[-8-] PTLQEMIQMAAE..D.A..NAKKF.....A.CM.AHDF.T.K.G...MT		
PDGFR		Y.I.E.CRY.D.VDY.HRN.HTF[-97-].SYTDL.GFS..V.N..DF.ASKNC.....A..ICEGKL.K.C.....A		

	Subdomain	VIII	IX	X
<i>let-23</i>		KILKHDADSITIKSGKVAIKWLAIEIFSKHCYTHASDVWAFGVTCWEIITFGQSPYQG-MSTDISHNFKLDGNRLSQPPNC		
EGFR		.L.GAEKEHYHAEG...P...M.L.SILHRI...Q...SY...V.LM...SK..D.-IPASE.SSI.EK.E..P...I.		
DER		.L.SS.SNEYKAAG..MP...L.CIRNRVF.SK.....I..LL...R.HEN-IPAKD.PDLIEV.LK.E..EI.		
SRC		RLIEDNEYTAR-QGA.FP...T.P.AALYGRF.IK...S..ILLT.LT.K.RV..P..VNREVLDQVER.Y.MPC..E.		
Abl		RLMTG.TYTAH-AGA.FP...T.P.SLAYNKFSIK.....LL...A.Y.MS..P..IDPSQVVEL.EKDY.MKR.EG.		
INSR		RDIVETDYRKGGK.LLPVR.M.P.SLKDGVF.TS..M.S...VL...TSLAEQ...L.NEQVLK.VM..GY.D..D..		
PDGFR		RDIMRDSNY.SKG.TYLP.L.M.P.SIFNSL..TL...S..ILL...F.L.GT..PELPMN.QFY.AI.R.Y.MA...AHA		

	Subdomain	XI
<i>let-23</i>		SQDLYQELLRCWMDPKSRPGFEILYERFKECKVP (1148)
EGFR		TI.V.MIMVK...I.AD...K.RE.IIE.SKMARD. (951)
DER		.L.I.CT..S..HL.AAM..T.KQ.TTV.A..ARD. (1121)
SRC		PES.HDLMQC..RKEPEE..T.Y.QAPLEDYFTST (523)
Abl		PEKV.ELMRA..QWN.SD..S.AEIHQA.ETMPQES (500)
INSR		PERVTDLMRM..QFN.NM..T.LEIVNLL.DDLHPS (1258)
PDGFR		.DEI.EIMQK...EEKFET..P.SQ.VLLERLLLEG (933)

		Cysteine-rich motif I										
<i>let-23</i>	31C5C1C25C114	C28C9C3C3C7C7C3C2C10	CC3C3C7C2C8C3C26C3C10C3C14C2C4C3C16C									66C27
DER	52C26C98	C29C2C3C7C7C3C4C7	CC3C3C7C2C12C26C3C10C3C2C3C16C									116
EGFR	18C11C26C98	C29C2C3C4C7C7C3C3C7	CC3C3C7C2C8C3C26C3C11C3C14C2C3C3C24C									107

		Cysteine-rich motif II-1										
<i>let-23</i>	C32C6C3C4C7C2C8C3C13C2C3C2C7C2C15C3C11C2C3C4C11C2											
DER	C28C6C3C4C7C2C8C3C13C2C3C2C7C2C8C3C10C2C3C2C11C2											
EGFR	C28C6C3C4C7C2C8C3C15C2C3C8C7C2C8C3C21C2C3C3C7C6											MB

		Cysteine-rich motif II-2										
<i>let-23</i>	C12C9C22C2C3C2C11C1C13C3C14C2C3C5C7C2C12C3C13C13											MB
DER	C13C6C22C2C3C2C9C2C8C3C12C6C2C7C2C21C3C17C9											MB

mutation that severely reduces *let-23* activity is epistatic to the gonad-independent multivulva mutation *lin-15(n309)* (R.V.A. and P.W.S., manuscript in preparation). That is, a lack of (or strong reduction in) *let-23* activity is not equivalent to a lack of the anchor-cell signal in a *lin-15(n309)* background, and we infer that *let-23* does not act in the anchor cell. Although *let-23* could act in other cells (see Fig. 7 legend), the simplest interpretation is that *let-23* is required for VPCs to respond to the anchor-cell signal.

On the basis of this genetic information and the molecular structure of the gene product, we propose that *let-23* encodes the receptor for the vulval inductive signal. In various tissue culture systems, EGF receptor elicits a mitogenic response in epithelial cells after binding of ligand (EGF or transforming growth factor- α (TGF- α))^{30,31}. We propose that the *let-23* product binds the anchor-cell signal molecule and subsequent transduction of that signal is required for VPCs to adopt vulval fates. Another receptor-like molecule acting during vulval development, the *lin-12* protein, has EGF-like (that is, ligand) repeats in its putative extracellular domain^{32,33}. But the phenotypes and genetic properties of *lin-12* mutants suggest that the *lin-12* product is unlikely to be a receptor for the inductive signal, although it might be a receptor for a signal acting between VPCs^{4,34}.

let-23 acts by way of *let-60 ras*

Genetic analyses have enabled the identification and ordering of the actions of many genes involved in *C. elegans* vulval determination^{4,11,13}. The finding that *let-23* encodes a growth factor receptor tyrosine kinase is relevant to the identification of another component of the vulval induction pathway, the *let-60* gene, which encodes a *ras* protein¹⁵. Because increased activity and increased dosage of *let-60 ras* suppresses *let-23* loss-of-function vulval defects, *let-60 ras* is believed to act downstream of *let-23* in the vulval determination pathway^{13,15}. Also, loss of *let-60 ras* function leads to the same phenotype as loss of *let-23* function, that is, a complete lack of vulval induction.

Several studies have suggested a connection between *ras* proteins and tyrosine kinase growth factor receptors. The effects of

serum, EGF and platelet-derived growth factor (PDGF) on cell growth are inhibited by injection of monoclonal antibodies directed against *ras* proteins³⁵ and the infection of cell lines with a *Ki-ras* oncogene abrogates growth requirements for EGF³⁶. A biochemical link between growth factor receptors and *ras* proteins has been suggested by studies of the GTPase activating protein GAP³⁷. GAP associates with and is phosphorylated by the receptor for PDGF^{38,39}, and phosphorylation of GAP can be stimulated by EGF in a cell line overexpressing EGF receptor⁴⁰. Furthermore, GAP has been implicated in regulating *ras* proteins because it catalyses the conversion of Ras-GTP to Ras-GDP^{41,42} and can inhibit morphological transformation by normal Ha-*ras*⁴³. So it has been hypothesized that GAP links growth factor receptors with *ras* proteins. These studies are consistent with genetic studies (see above) that conclude that the chief effect of the *let-23* kinase is exerted through *let-60 ras*.

Our model for *let-23* and *let-60 ras* action during vulval induction is summarized in Fig. 7. Genetic analysis of the vulval pathway in combination with the molecular characterization of two of the genes involved has provided support for a link *in vivo* between a protein of the growth factor receptor tyrosine kinase family, *let-23*, with a *ras* protein, *let-60*. Because mutations in *let-60 ras* lead to similar lethal and male tail phenotypes as *let-23* mutations, and because an increase in *let-60* activity suppresses *let-23* lethality (ref. 13 and R.V.A. and P.W.S., manuscript in preparation), the proteins encoded by these two genes most probably operate together in more than one *C. elegans* developmental pathway. □

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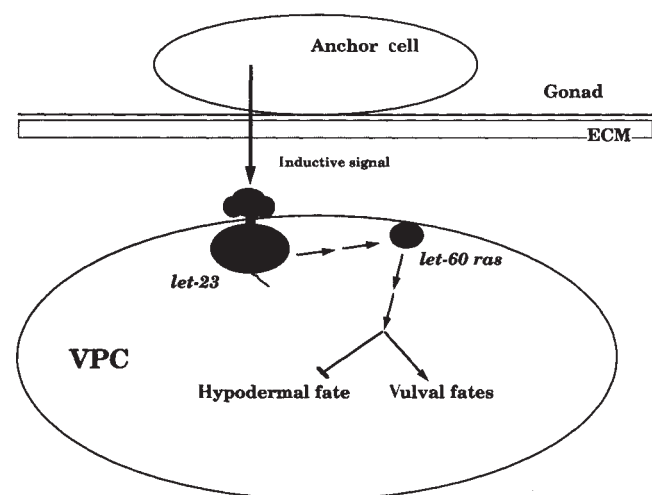


FIG. 7 Hypothetical function for *let-23* and *let-60 ras* during vulval induction. We envisage that *let-23* is embedded in the plasma membrane of the VPCs and that *let-60 ras* is associated with the membrane. The *let-23* receptor binds the inductive signal, which apparently diffuses from the anchor cell^{6,7}, and subsequently activates *let-60 ras*. After activation by *let-23*, *let-60 ras* causes VPCs to execute vulval fates instead of hypodermal fates^{13,15}. ECM, extracellular matrix. (Evidence suggests that *lin-15* acts in cells other than VPCs or anchor cell¹⁷. Therefore *let-23* and/or *let-60 ras* might operate in cells other than VPCs. Nonetheless, all data so far are compatible with *let-23* and *let-60 ras* acting in the VPCs and *lin-15* acting in other cells to prevent the action of *let-23/let-60* in some VPCs by way of an intercellular signal.)

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A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas

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A gene has been identified that is expressed specifically in stromal cells surrounding invasive breast carcinomas. On the basis of its sequence, the product of this gene, named stromelysin-3, is a new member of the family of metalloproteinase enzymes which degrade the extracellular matrix. The suggestion is that stromelysin-3 is one of the stroma-derived factors that have long been postulated to play an important part in progression of epithelial malignancies.

In most cancers lethality is the result of local invasion and metastasis of neoplastic cells from the primary tumour to other tissues (tumour progression). The dissociation of the metastatic cells depends on changes both to the extracellular matrix (ECM) surrounding the primary tumour and to the adhesive properties of the tumour cells themselves (ref. 1 and references therein). Tumour development also depends on angiogenesis, which again requires ECM degradation². Interactions between neoplastic cells and their environment thus play a crucial part in the development of malignancy^{3–7}. Although changes to two sets of cellular genes, the proto-oncogenes and the tumour-suppressor genes, have been shown to lead by a multistep process to cellular transformation^{5,8}, the molecular mechanisms leading to tumour progression are much less clear (see refs 1, 2, 6 and 9 for reviews).

Most human malignant tumours are carcinomas, and among non-smoking women, breast cancer is the leading cause of mortality due to cancer¹⁰. Several oncogenes have been reported to be altered in malignant breast cells and tumours, but no particular pattern of oncogene or tumour-suppressor gene expression has so far been shown to be consistently associated with breast cancer¹¹. The neoplastic cells of breast tumours, however, are often embedded in an adipose and mesenchymal stroma, which may be important in control of their proliferation and ability to metastasize. There is indeed evidence that stromal cells can influence the growth of normal mammary epithelium as well as epithelial carcinogenesis in the mammary gland^{12–16}. The existence of 'activated'^{14,17} or 'abnormal'¹⁸ fibroblasts in malignant breast tumours has been postulated, and it has been

suggested that breast cancer could represent "a partial escape from dependence on a stromal requirement or an abnormally strong response to a stromal component"^{13,14}.

Within this context, the present study was designed to identify genes specifically overexpressed in breast carcinomas. One such gene was found to have a sequence suggesting that it encodes a new member of the family of metalloproteinases, enzymes which degrade the ECM (ref. 19 and refs therein). In contrast to other metalloproteinase genes tested, the new gene, named stromelysin-3 gene, is expressed in all invasive breast cancers analysed so far, suggesting that its expression may be one of the molecular events required for breast cancer progression. Most interestingly, stromelysin-3 gene expression is restricted to the stromal cells immediately surrounding the neoplastic cells of the invasive, but not the *in situ*, component of breast carcinomas.

Cloning stromelysin-3 complementary DNA

A subtracted breast cancer complementary DNA library was constructed from a surgical specimen (C1), rather than from an established breast cancer cell line, in order to have both neoplastic and stromal cell components (see Fig. 1 legend). The library was differentially screened (Fig. 1 legend) and four genes were identified that were overexpressed in the carcinoma, but not in a fibroadenoma (F1, taken as non-malignant breast tissue; Fig. 1). Gene D was chosen for further study because of its lack of expression in several normal tissues (see below and data not shown).

Using the D cDNA insert as a probe, other clones were identified and used to obtain the full-length cDNA sequence shown in Fig. 2. The open reading frame is predicted to encode a 488-residue protein, with the chosen initiation codon supported by the presence of a putative hydrophobic leader peptide in the immediately downstream sequence. Comparison of the predicted protein sequence with the Swissprot data library (release 14) suggested that the putative new protein belongs to the family of secreted matrix metalloproteinases (MMPs; Fig. 3a). The protein has an hydrophobic N-terminal sequence (underlined in Fig. 2) which is a candidate leader sequence and contains the highly conserved sequence Pro-Arg-Cys-Gly-Val-Pro-Asp (PRCGVPD, single-letter amino-acid code; residues 78–84) characteristic of the propeptide domain of MMPs, as well as the putative MMP zinc-binding site (residues 212–225)^{19,20}. By analogy with other MMPs the mature protein is predicted to start at phenylalanine 98 (ref. 21) (Fig. 3a). After

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optimal alignment the putative mature protein shows 40% sequence similarity with stromelysin-1 (refs 21–23), 38% with stromelysin-2 (ref. 23) and 36% with type I collagenase^{21,24} (Fig. 3b).

In the absence of data on the substrate specificity of this new MMP, we have named it stromelysin-3 (ST3), although its similarity with stromelysin-1 is much less than that between stromelysin-1 and stromelysin-2 (79%), and even lower than that between stromelysin-1 and type I collagenase (53%) (Fig. 3b). It is interesting that upstream of the Pro-Arg-Cys-Gly-Val-Pro-Asp sequence there is no similarity between ST3 and the other MMPs, and, moreover, ST3 has an additional unique short sequence (residues 88–97) located precisely at the proprotein cleavage site of type I collagenase and stromelysins²¹. ST3 does, however, share with type I collagenase and the other stromelysins the absence of the fibronectin-like domain that characterizes type IV collagenases^{25,26}.

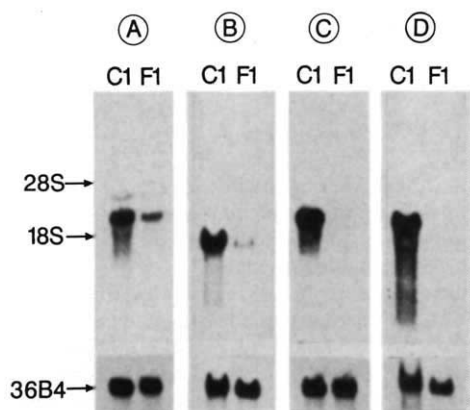


FIG. 1 Northern blot analysis of total RNA from C1-breast carcinoma and F1-fibroadenoma using cDNA probes of four genes (A–D) exhibiting higher levels of expression in the carcinoma than in the fibroadenoma. Each lane contained 8 µg total RNA. The filters were reprobed using the 36B4 probe which corresponds to ubiquitously expressed gene⁴⁴.

METHODS. Total RNA was prepared, using the guanidium-chloride method, from surgical specimens stored in liquid nitrogen and poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography. A breast cancer-enriched cDNA library was constructed using cDNA prepared from an oestrogen receptor-negative, grade II, ductal carcinoma (referred to as C1), in which stromal cells represented ~50% of the total cell population. Before cloning, the single-stranded cDNA was subtracted with an excess of poly(A)⁺ RNA from a breast fibroadenoma (referred to as F1), and the single-stranded enriched material was purified by hydroxylapatite chromatography^{45,46}. The breast cancer-enriched cDNA was made double-stranded and cloned into the *Eco*RI site of the λ gt10 vector. Three million recombinant phages were obtained, and ~50,000 were differentially screened using replica nylon filters (Biodyne A, Pall Corporation) from plates containing ~5,000 cDNA clones. (+) and (–) probes were made using C1-breast cancer cDNA and F1-breast fibroadenoma cDNA, respectively. Both probes were subtracted^{45,46} with an excess of total human liver RNA before labelling with ³²P using random priming synthesis. Hybridizations were carried out for two days under stringent conditions (50% formamide, 42 °C) and washing was performed in 2xSSC, 0.1% SDS, at 22 °C, followed by 0.1xSSC, 0.1% SDS at 55 °C. 130 differentially labelled plaques were selected for a second screening. The cDNA inserts of five 'differential' plaques taken at random were purified by amplification in the polymerase chain reaction (PCR), ³²P-labelled, and hybridized to all of the 'differential' plaques to identify related clones. This procedure was repeated several times with differential plaques taken at random, yielding finally four genes referred to as A to D, which exhibited higher levels of expression in C1-carcinoma than in F1-fibroadenoma. The northern blots for C1-breast cancer and F1-breast fibroadenoma were prepared using total RNA (8 µg) separated by electrophoresis in 1% agarose gels containing formaldehyde and transferred to Hybond-N filters (Amersham). The blots were stained with methylene blue before prehybridization to check for the integrity and amounts of transferred RNA. Hybridization (18 h) and washing were performed under standard conditions, as described above, using ³²P-labelled cDNA inserts corresponding to A–D genes.

CCGGGGCGGATGGCTCCGGCCGCTGGCTCCGAGCGCGCGCGCCCTCTGCCC	60
M A P A A W L R S A A R A L L P	17
CCGATGCTGCTGCTGCTCCAGCCGCGCGCTGCTGGCCGGGCTCTGCCCGGAC	120
P M L L L L L Q P P P L L A R A L P P D	37
GTCCACCACTCCATGCCGAGGAGGGGCCACCCCTGGCATGACCCCTGCCAGT	180
V H H L A E R R G P Q P W H A A L R G S	57
AGCCCGGACCTGCCCTGCCACGAGGAAGCCCCCGGCTGCCAGCAGCTCAGGCCT	240
S P A P A P A T Q E A P R P A S S L R P	77
CCCCGCTGGGCTGCCGACCCATCTGATGGGCTGAGTGGCCGCAACGACAGAAGG	300
P R C G V P D P S D G L S A R N R Q K R	97
TTCGTGCTTCTGGCGGCTGGGAGAGCGGACCTCACTACAGGATCCTTCGGTTC	360
F V L S G G R W E K T D L T Y R I L R F	117
CCATGGCAGTTGGTGACGAGCAGGTGCGGACAGCATGGCAGAGGCCCTAAAGGTATGG	420
P W Q L V Q E Q V R D L P F Q T M A E A L K V W	137
AGCGATGTGACGCCACTCACCTTTACTGAGGTGACAGAGGCGCTGCTGACATCATGATC	480
S D V T P L T F T E V H E G R A D I M I	157
GACTTCGCGAGTACTGGGATGGGACGACCTGCCGTTTGTAGTGGCGCTGGGGCATCTG	540
D F A R Y W D G D D L F P D C R G V Q K R	177
GCCCATGCTTCTTCCCAAGACTCACCGAGAAGGGGATGCCACTTCGACTATGATGAG	600
A H A F F P K T H R E G D V H F D Y D E	197
ACCTGGACTATGGGGATGACAGGCGACAGCTGCTGAGGTGGCAGCCCATGAATTT	660
T W T I G D D Q G T D L L Q V A A H E F	217
GGCCACGTGCTGGGCTGACGACACACAGCAGCCAGGCCCTGATGTCGCGCTTCTAC	720
G H V L G L Q H T T A A K A L M S A F Y	237
ACCTTCGCTACCCACTGAGTCTCAGCCAGATGATGACAGGCGCTTCAACACTATAT	780
T F R Y P L S L S P D D C R G V Q K R	257
GGCCAGCCTGGGCCACTGTCACTCAGGACCCAGCCCTGGGCGCCAGCTGGGATA	840
G Q P W P T V T S R T P A L G P Q A G I	277
GACCAATGAGATTGACCGCTGGAGCGAGCGCCCGCAGATGCTGTGAGGCCCTCC	900
D T N E I A P L E P D A P D A C E A C	297
TTTGACGGCTTCCACCATCCGAGCGAGCTCTTTTCTCAAAGCGGCTTTGTGGG	960
F D A V S T I R G E L F F F K A G F V W	317
CGCTTCGCTGGGGCCAGCTGACGCGCGCTACCCAGCATGGGCTCTGCCAGCTGGCAG	1020
R L R G G Q L Q P G Y P A L A S R H W Q	337
GGACTGCGCCAGCTGTGGAGCTGCTTCGAGGATGCCAGGCGCACATTGTGTTCTTC	1080
G L P S P V D A A F E D A Q G H I W F F	357
CAAGTGTCTAGTACTGGGTGTACGACGGTGAAGAAGCTCTGGGCGCCGACCCCTC	1140
Q G A Q Y W V Y D G E K P V L G P A P A L	377
ACCGAGCTGGGCTGGTGGCTTCCGCTCATGCTGCTTGGTGTGGGCTCCGAGAAG	1200
T E L G L V R F P V H A A L V W G P E K	397
AACAAGTCTACTTCTCCGAGGAGGACTACTGGCTTTCCACCCAGCAGCCGCGCT	1260
N K I Y F F R G R D Y W R F H P S T R A R	417
GTAGACGTCCGCTGCCCGCCAGGCGCACTGCTGAGGAGGGGCTCTGAGATCGAC	1320
V D S F V P R R A A T D W R G G V P S E I D	437
GCTGCTTCCAGGATGCTGATGGCTATGCTTCTCTGCGCGCGCCCTTACTGGAAG	1380
A A F Q A D A D G Y A Y F L R G R L Y W K	457
TTTGACCTGTGAAGGTGAAGGCTTGAAGGCTTCCCGCTCTCGTGGGCTGACTTC	1440
D F P V K V K A L E G F P R L V G P D F	477
TTTGCTGTGCCAGCCTGCCAACACTTCTCTGACCATGGCTTGGATGCCCTCAGGG	1500
F G C A E P A N T F L	488
TGCTGACCCCTGCCAGGCGCAGAAATCAGGCTAGACACCATGGCCATCTTTGTGGCTG	1560
TGGGCACCGAGCATGGGACTGAGCCCATGCTCTCTGAGGGGATGGGGTGGGATCAAC	1620
CACCATGACAACTGCCGGGAGGGCCAGCGAGTCTGGTCACTGCCAGCACTGTCTCA	1680
GAGTGGGAGGGAGGCTTTGGCATGACTTAAGAGGAAGGGCAGCTTTGGGACCCGCTATG	1740
CAGGCTTGGCAACCTGGCTGCCCTGTCTCATCCCTGTCCTCAGGCTAGCAGCATGGC	1800
AGGACTGGGGAGCTGGAGTGTCTTCTGTATCCCTGTGTGAGGTCTCTTCCAGGGG	1860
TGACTGAGCAAGGGTGTGGGGCCCATGGCTTCAAGCCCTGCTGAGCACTGGG	1920
TGTAGGGGAGGCTTCTGAGGTGAGGCTTCTGAGGTGCTGCTGCTGCTGCTGCTG	1980
CTGGTGAACATCTGGAATCTGTCTTCCAGAAATCCAGGCCAAAAGTTACAGTCAAA	2040
TGGGAGGGGTATTCTTCATGAGGAGACCCAGGCCCTGGAGGTGCAACATACCTCAA	2100
TCTGTCCAGGCGGATCTCTGAGGCCCTTTTCGAGCAGTGTATCTTCCAAAGCC	2160
ATTGTAATGTGTACAGTGTGTATAAACCCTTCTTCTTTTCTTTTAACTGAG	2220
GATTGCTTAAACACAGTGTGTTTCTTAAAAAAA...	

FIG. 2 Nucleotide sequence of stromelysin-3 cDNA and deduced amino-acid sequence. Nucleotide residues are numbered in the 5' to 3' direction and deduced amino acids in the open reading frame are designated by their one-letter codes. Starting from the 5' end, the underlined nucleotide sequences correspond to the putative signal peptide⁴⁷ (two potential cleavage sites are marked by arrows), the PROGVPD sequence characteristic of prometallproteinases⁴⁹, the conserved histidine residues of the zinc-binding domain¹⁹ and the poly(A) addition signal sequence.

METHODS. A cDNA insert corresponding to the 3' part of D cDNA (250 base pairs including a 19-bp poly(AT) region) was labelled with ³²P by random priming synthesis and used to screen a non-subtracted λ gt10 cDNA library generated from C1-breast tumour poly(A)⁺ RNA. Several independent clones were identified and subcloned in M13 sequencing vector. DNA sequence was determined by the dideoxy-chain-termination method using Sequenase and the deaza-dGTP reagent kit (US Biochemicals). The sequence was analysed using the PC/GENE software package.

Overexpression in breast carcinomas

ST3 gene expression was studied in 30 breast carcinomas and 5 fibroadenomas, and compared with that of several other members of the MMP gene family (Fig. 4 and data not shown). ST3 RNA was found in all breast carcinomas tested, both those negative (C1–C4) and those positive (C5–C10) for the oestradiol receptor, but not in fibroadenomas (except for a low level of expression in F2; Fig. 4a).

The MMPs tested could be divided into two classes by their pattern of expression. 72K type IV collagenase (COIV 72K), stromelysin-1 and -2 (ST1/2) and pump-1 (PU1) genes were expressed in both malignant and benign tumours. ST3, type I collagenase (COI) and 92K type IV collagenase (COIV 92K) genes on the other hand were overexpressed in only the breast carcinomas. The genes of this second class, however, differed in their precise expression patterns, and only ST3 RNA was clearly detected in all the carcinomas, suggesting that this MMP in particular may be associated with breast cancer progression. ST3 gene expression was also found in breast cancer metastatic, but not in normal, lymph nodes (Fig. 5a).

No ST3 gene expression, however, could be detected in eight breast cancer cell lines, irrespective of their oestradiol receptor status (Fig. 5b). Nor could ST3 RNA be detected in several normal human adult tissues (Fig. 5c), with the notable exceptions of uterus and placenta. Low levels of ST3 RNA were detected in colon, ovary, kidney and lung cancers, whereas high levels were seen in larynx cancer (data not shown).

Expression specific to stromal cells

The absence of ST3 gene expression in breast cancer cell lines suggested that it might be expressed in tumour stromal cells rather than in the neoplastic cells themselves. To test this we carried out *in situ* hybridizations (Fig. 6 legend) on sections of six carcinomas (tumours C1, C3, C5, C9 and C10 as in Fig. 4, and C11, a grade II carcinoma positive for the oestradiol receptor. In all cases we found ST3 RNA in only the stromal cells

surrounding the islands of malignant epithelial cells of the invasive components of the tumours (Fig. 6a and b for C1; c, d, e and f for C3; g and h for C10; i and j, right side for C11; data not shown for C5 and C9). ST3 gene expression was also restricted to stromal cells surrounding metastatic epithelial cells in the case of the metastatic lymph nodes from patient C5 (data not shown).

It is striking that in all cases the malignant epithelial cells themselves are not labelled, and that the highest levels of expression are found in the stromal cells closest to the malignant cells. In marked contrast, no significant expression could be detected in the stromal cells surrounding the carcinoma *in situ* lesions (g and h for C10; i and j, left side for C11) or at a distance from the cancerous cells, or in the stromal cells surrounding normal ducts and ductules (e and f). No discrete foci of ST3 RNA were detected in a section of the F2 fibroadenoma that was weakly positive for ST3 RNA on northern blots (Fig. 4a and data not shown). Both fibroblasts and myofibroblasts are present in the stroma of invasive breast carcinomas^{17,27}, but by *in situ* hybridization we could not determine whether only one or both of these cell types expressed the ST3 gene.

Growth factor stimulation

These results suggested that ST3 gene expression in stromal cells is induced by a diffusible factor secreted by the neoplastic cells. Growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), as well as some cytokines and tumour promoters such as TPA (12-*O*-tetradecanoylphorbol-13-acetate) activate transcription of MMP genes^{19,28}. It has also been reported that tumour cells from several sources produce a factor(s) that stimulates collagenase I production by human fibroblasts²⁹. PDGF, FGF and transforming growth factor- α (TGF- α) are secreted by breast cancer cells *in vitro*^{11,13,14}. We therefore tested whether ST3 gene expression could be modulated by growth factors and TPA. We found that PDGF, EGF, basic FGF (bFGF) and TPA

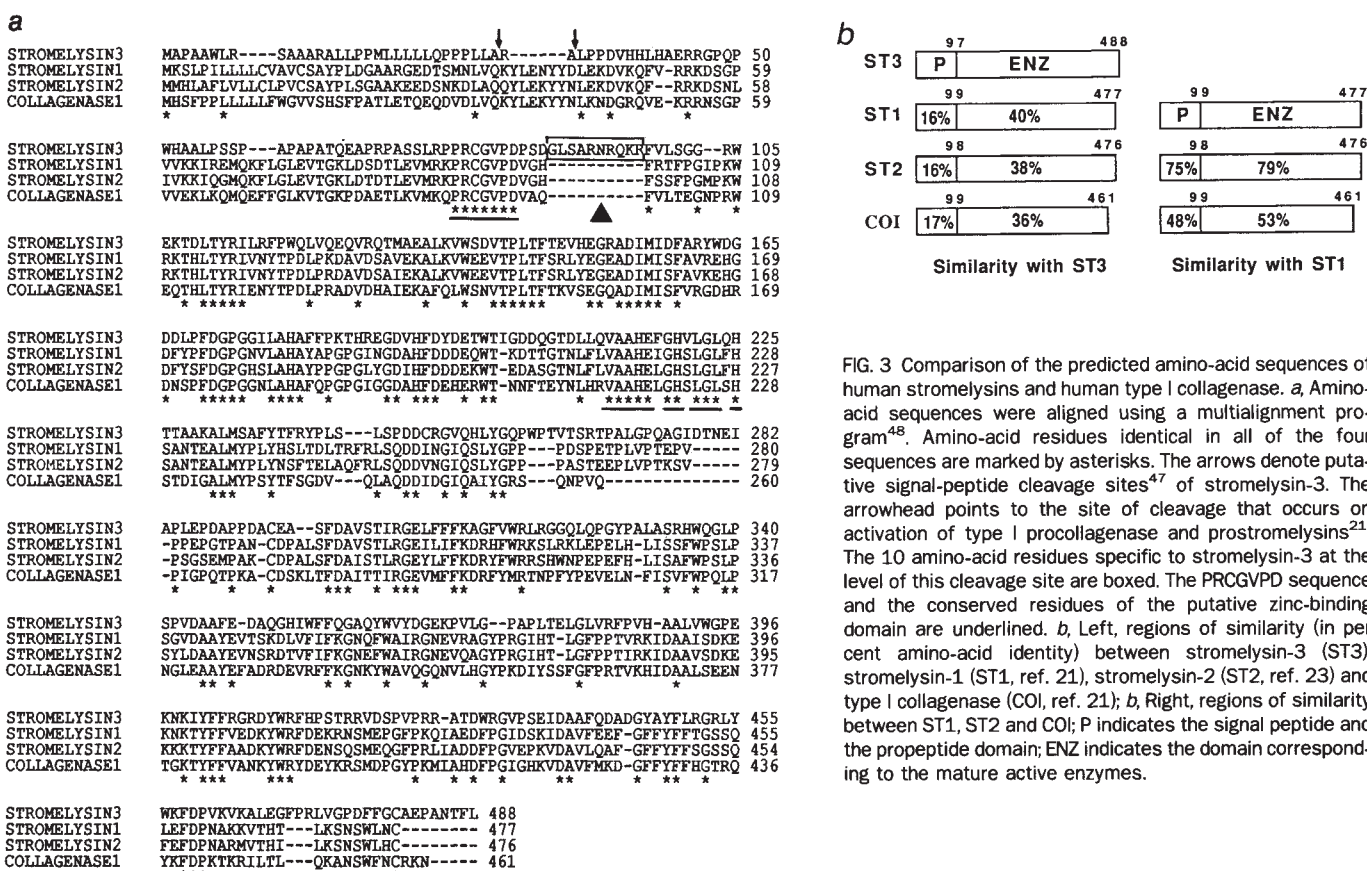
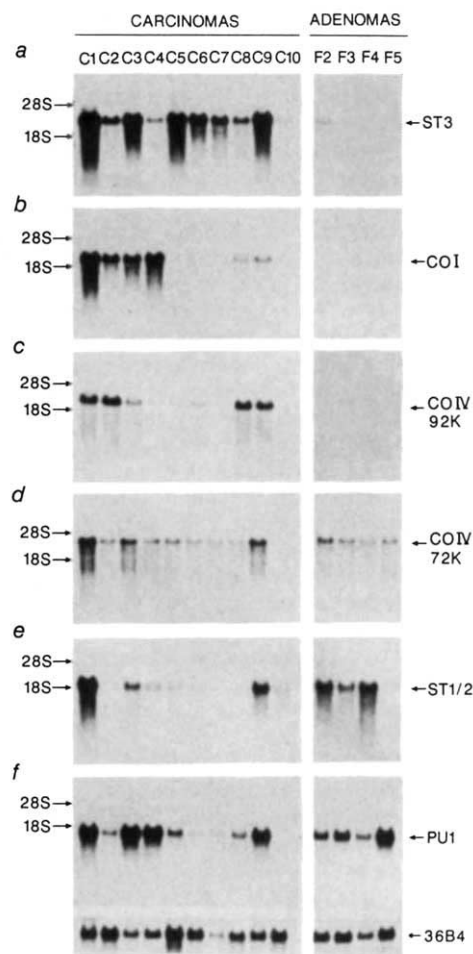


FIG. 3 Comparison of the predicted amino-acid sequences of human stromelysins and human type I collagenase. **a**, Amino-acid sequences were aligned using a multialignment program⁴⁸. Amino-acid residues identical in all of the four sequences are marked by asterisks. The arrows denote putative signal-peptide cleavage sites⁴⁷ of stromelysin-3. The arrowhead points to the site of cleavage that occurs on activation of type I procollagenase and prostromelysins²¹. The 10 amino-acid residues specific to stromelysin-3 at the level of this cleavage site are boxed. The PRGCVDP sequence and the conserved residues of the putative zinc-binding domain are underlined. **b**, Left, regions of similarity (in per cent amino-acid identity) between stromelysin-3 (ST3), stromelysin-1 (ST1, ref. 21), stromelysin-2 (ST2, ref. 23) and type I collagenase (COI, ref. 21); **b**, Right, regions of similarity between ST1, ST2 and COI; P indicates the signal peptide and the propeptide domain; ENZ indicates the domain corresponding to the mature active enzymes.

FIG. 4 Northern-blot analysis of human metalloproteinase RNAs in breast tumours. *a*, Stromelysin-3 RNA (ST3); *b*, type I collagenase RNA (COI); *c*, 92K type IV collagenase RNA (COIV 92K); *d*, 72K type IV collagenase RNA (COIV 72K); *e*, stromelysin-1 and -2 RNAs (ST1/2); *f*, pump-1 RNA (PU1). Total RNA was prepared from four oestrogen receptor-negative breast carcinomas (C1, grade II; C2, C3 and C4, grade III), six oestrogen receptor-positive breast carcinomas (C5, C8 and C9, grade II; C6 and C7 grade III; C10, grade I) and four breast fibroadenomas (F2–F5). Each lane contained 8 μ g RNA. The 36B4 signal corresponds to the RNA of a control gene (see Fig. 1).

METHODS. Several northern blots were prepared in parallel with identical RNA samples, as described in Fig. 1, and hybridized with either of the following cDNA probes: *a*, 1.6-kilobase(kb) insert covering the 3' part of ST3 cDNA; *b*, COI cDNA; *c*, ST2 cDNA (which cross-hybridizes with ST1 RNA); *d*, PU1 cDNA (COI, ST2 and PU1 probes kindly provided by R. Breathnach, ref. 23); or 80-mer antisense oligonucleotide probes corresponding to *c*, COIV 92K (nucleotides 2,144–2,223, ref. 26) and *d*, COIV 72K (nucleotides 1,937–2,016, ref. 25). The cDNA probes were labelled with 32 P using random priming synthesis ($\sim 5 \times 10^8$ c.p.m. μ g $^{-1}$) and the oligonucleotides were labelled using 5'-end kination ($\sim 10^6$ c.p.m. μ g $^{-1}$). Hybridizations were carried out under stringent conditions (42 °C, 50% formamide; $\sim 10^6$ c.p.m. ml $^{-1}$). The filters were then washed in 2 $\times$ SSC, 0.1% SDS at 22 °C, followed by 0.1 $\times$ SSC, 0.1% SDS at 55 °C. Autoradiography was for: *a*, 18 h; *b*, 20 h; *c*, *d* and *e*, 4 days; *f*, 2 days, all at –80 °C with an intensifying screen.



could all increase ST3 gene expression in human fetal diploid fibroblasts, the strongest stimulation being with bFGF (Fig. 5*d*).

Embryonic expression

ST3 gene expression in fetal fibroblasts stimulated by growth factor prompted us to investigate whether the gene is normally expressed during embryonic development. We detected ST3 RNA in several discrete regions of an 8-week human embryo, notably in the interdigital region of the limb buds (Fig. 6*k, l*, and data not shown), an area associated with programmed cell death at this stage of embryogenesis. The labelling was observed in the embryonic mesoderm underlying the primitive epiderm, which was itself unlabelled. The mesenchymal cells at a distance from the epiblast were also unlabelled. ST3 gene expression during normal embryonic development, in an area where tissue remodelling is well documented, supports the suggestion that ST3 expression in breast carcinomas plays a part in the ECM remodelling associated with tumour progression.

Discussion

We have described here what we believe to be a novel member of the MMP family which is expressed in breast carcinomas. All known MMPs degrade at least one component of the ECM, are secreted in latent form and require an activation step such

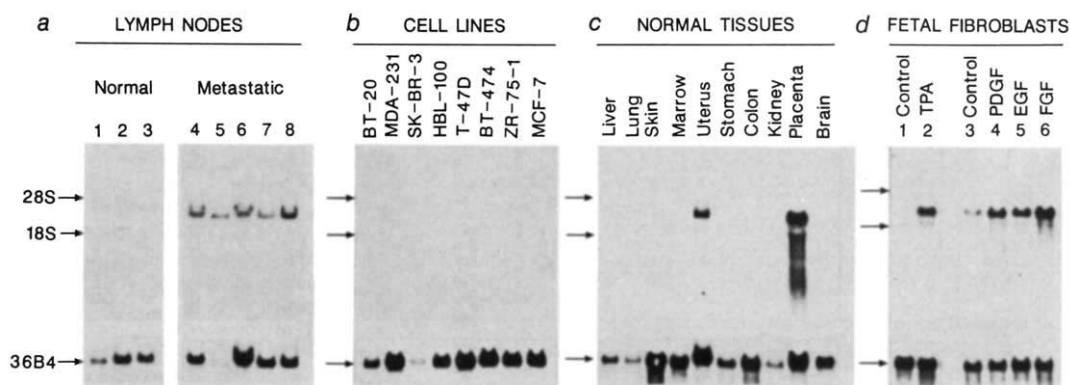


FIG. 5 Northern blot analysis of stromelysin-3 RNA in various cell lines and tissues. *a*, Three normal and five metastatic axillary lymph nodes from patients with breast cancers. *b*, Four oestrogen receptor-negative (BT-20, MDA-231, SK-BR-3, HBL-100) and four oestrogen receptor-positive (T-47D, BT-474, ZR-75-1, MCF-7) breast cancer cell lines. *c*, Ten normal human tissues. *d*, HFL-1 human fetal diploid fibroblasts (ATCC CCL 153) cultured in serum-free medium (1 and 2), in the absence (1) or presence (2) of TPA (10 ng ml $^{-1}$) or cultured in serum-free medium supplemented with 20 μ g ml $^{-1}$ insulin (3 to 6), in the absence (3) or presence of PDGF (4; 20 ng ml $^{-1}$, British Bio-Technology), EGF (5; 20 ng ml $^{-1}$, Collaborative Research) or bFGF (6; 10 ng ml $^{-1}$, kindly provided by B. Pettmann; ref. 49).

In *a*, each lane contained 10 μ g total RNA with the exception of lane 5 (2 μ g) and lane 6 (20 μ g). In *b* and *c*, each lane contained 8 μ g total RNA, and in *d*, each lane contained 5 μ g cytoplasmic RNA.

METHODS. In *a*, *b* and *c*, the blots were made and processed as indicated in Fig. 4 for stromelysin-3. In *d*, confluent HFL-1 fibroblasts were kept in serum-free DMEM culture medium. After 24 h, fresh medium was added and some cultures were supplemented with TPA or growth factors, as indicated above. After 24 h of culture, the cells were collected and cytoplasmic RNA prepared. The blots were then prepared and processed as indicated in Fig. 4 for stromelysin-3, but the autoradiography was for three days.

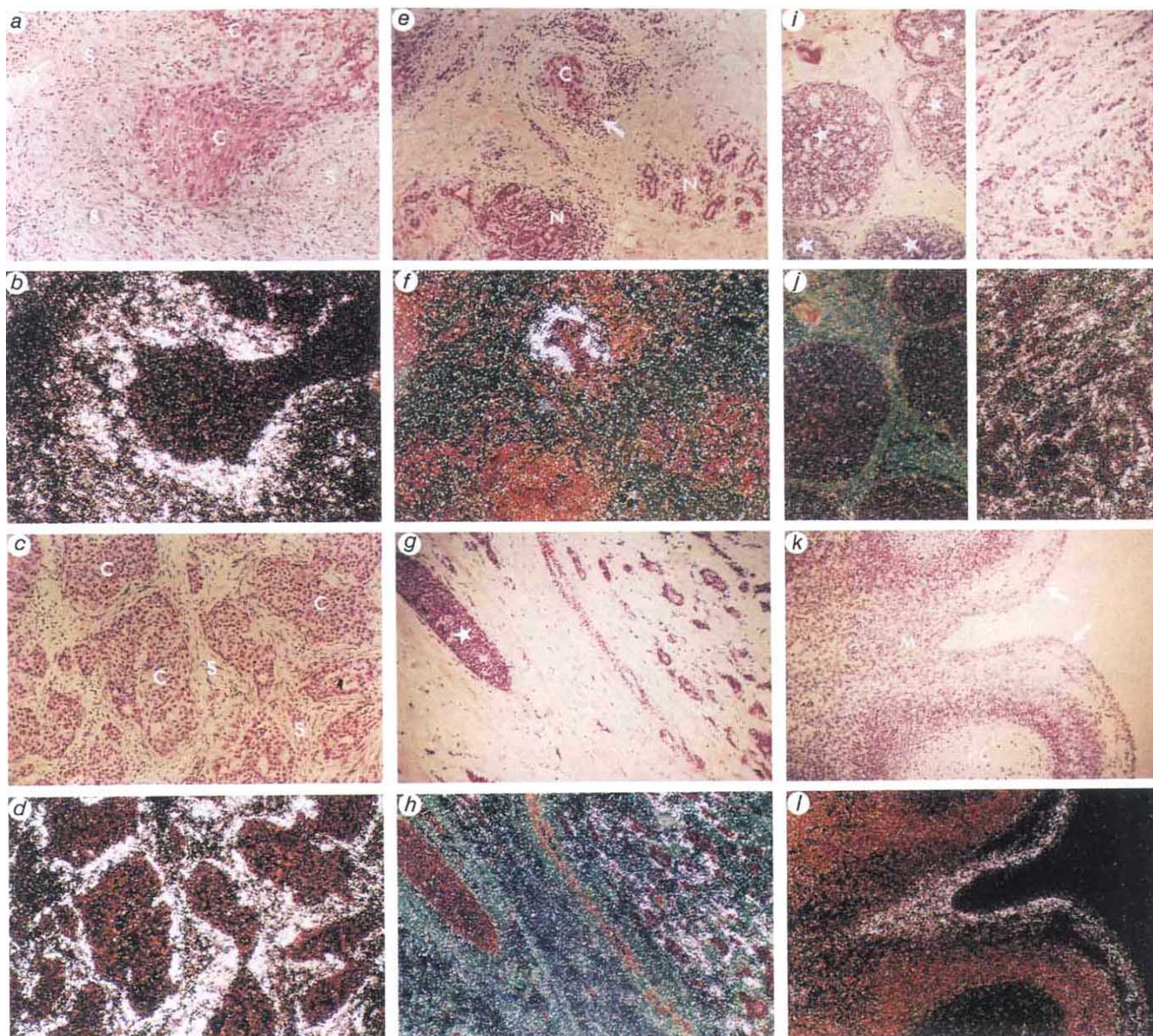


FIG. 6 Stromelysin-3 RNA transcripts in sections of breast carcinomas and embryo limb bud. *a, c, e, g, i, k*, Bright-field micrographs of tissue sections ($\times 100$) stained with haematoxylin. *b, d, f, h, j, l*, Dark-field micrographs of the same sections after *in situ* hybridization with an antisense stromelysin-3 (ST3) cRNA probe. *a, b*, Grade II ductal breast carcinoma (tumour C1, see Fig. 4): infiltrating cancer cells (C) are surrounded by a stroma rich in fusiform cells (S); ST3 RNA transcripts are most abundant in the stromal cells immediately surrounding the neoplastic epithelial cells. *c, d*, Grade III ductal breast carcinoma (tumour C3, see Fig. 4): multiple islands of infiltrating breast cancer cells (C) are surrounded by stromal cells; note that the expression of the ST3 gene is weaker in the central part of most of the stromal trabeculae (S), that is, in the region which is the farthest from the neoplastic cells. *e, f*, Ductal carcinoma (tumour C3, see Fig. 4) together with two normal lobules (N); ST3 RNA transcripts were detected exclusively in the stroma apposing the infiltrating cancer cells (C), with the exception of a small area rich in lymphocytes (arrow). *g, h*, Grade I ductal carcinoma (tumour C10, see Fig. 4): ST3 RNA transcripts can be detected above background in the stromal cells surrounding the infiltrating (upper corner, right) but not the *in situ* (asterisk) breast cancer cells. *i, j*, Ductal carcinoma

(tumour C11, ER-positive, grade II, carcinoma). Left, *in situ* carcinoma (asterisks) no ST3 RNA transcripts can be detected in the stromal cells. Right, infiltrating neoplastic cells surrounded by stromal cells expressing the ST3 gene. *k, l*, Interdigital region of a 8-week-old human embryo limb bud: ST3 RNA transcripts are detected in the mesoderm underlying the primitive epiderm, most notably in the interdigital area (M). Note that the primitive epiderm (arrows), the cartilage in formation (PC), and the surrounding mesoderm are not labelled.

METHODS. *In situ* hybridization was carried out as described in ref. 50. Deparaffinized and acid-treated sections (6- μ m thick) were treated with proteinase K and hybridized overnight with 35 S-labelled antisense transcripts from a stromelysin-3 cDNA insert (467 bp extending from nucleotides 1,128 to 1,594) subcloned in Bluescript II (Stratagene). Hybridization was followed by RNase treatment (20 μ g ml $^{-1}$, 30 min, 37 $^{\circ}$ C) and two stringent washings (2 $\times$ SSC, 50% formamide, 60 $^{\circ}$ C, 2 h), before autoradiography using NTB2 emulsion (Kodak). Autoradiography was for 15 days. No significant labelling above background was observed under similar conditions using a sense riboprobe (not shown).

as proteolysis^{19,20}. The ST3 sequence differs from those of previously characterized MMPs in ways, suggesting that it may be distinct in its maturation and activation properties, its substrate specificity and its interactions with tissue metalloproteinase inhibitors³⁰⁻³².

MMPs are involved in several physiological and pathological processes in which ECM remodelling and cell migration are implicated (see refs 1, 2, 19, 33 and 34 for reviews). Furthermore MMP inhibitors, including tissue inhibitors of metalloproteinases, inhibit tumour invasion and angiogenesis³⁵⁻³⁸. The specific expression of ST3 gene in the invasive, but not *in situ*, component of all primary breast carcinomas we have analysed, in metastatic nodes and in tissues where extensive ECM remodelling is known to occur (uterus, placenta and embryonic limb bud) suggests that ST3 may play an important part in breast cancer progression. ST3 may be involved in the lytic processes likely to be associated with invasive tumour growth, and perhaps also in the desmoplasia that is associated with most invasive breast cancer lesions^{4,17}, and which may represent a host reaction to prevent further spread of malignant cells²⁷.

The restricted expression of ST3 gene in stromal cells immediately surrounding the neoplastic islands strikingly supports previous suggestions that there are important interactions between tumour epithelial cells and surrounding stromal cells in breast cancer^{4,17}. The pattern of ST3 gene expression observed suggests that the neoplastic cells produce a concentration gradient of a diffusible factor that induces transcription of the ST3 gene in the surrounding stromal cells, thus supporting the suggestion that fibroblasts in the neighbourhood of the neoplastic cells are 'activated' by some inductive stimulus emanating from the cancer cells^{4,17}. Our results show that several growth factors secreted

by breast cancer cells *in vitro*^{11,13,14} could constitute such an inductive stimulus. It is interesting that the breast cancer cell lines we have studied all fail to express the ST3 gene, even though some secrete and have receptors for EGF/TGF- α and FGF^{11,14}.

There is an interesting parallel between expression of the ST3 gene and that of the tenascin gene in breast carcinoma. The ECM glycoprotein tenascin seems to play an essential part in epithelial-mesenchyme cell interactions and cell migration during development, including that of the mammary gland during organogenesis (refs 39, 40 and references therein). Tenascin has consistently been found to be overexpressed in the stroma of malignant breast tumours^{39,41} and seems to be induced in the stromal fibroblasts by the neoplastic epithelium (reviewed in ref. 40). Tenascin is a relatively poor substrate for attachment of mammary tumour epithelial cells and could allow them to become invasive³⁹. Our results raise the possibility that ST3 acts with tenascin during the invasive phase of breast cancer.

In conclusion, we have shown that the use of whole human malignant tumour samples can lead to the identification of genes whose expression is linked to tumour progression, and which would not necessarily be found by studying neoplastic cells cultured *in vitro*. ST3, seemingly the first example of a protease specifically produced by breast cancer stromal cells, may represent a potential target for breast cancer therapy. It remains to be seen how ST3 participates in tumour progression, in the context of other proteinases, proteinase inhibitors and ECM proteins, which are all present in the surroundings of the neoplastic cells, and some of which are produced by these cells (for example, cathepsin D and type IV collagenase, see refs 42 and 43). □

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The cosmological constant and cold dark matter

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THE cold dark matter (CDM) model¹⁻⁴ for the formation and distribution of galaxies in a universe with exactly the critical density is theoretically appealing and has proved to be durable, but recent work⁵⁻⁸ suggests that there is more cosmological structure on very large scales ($l > 10 h^{-1}$ Mpc, where h is the Hubble constant H_0 in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$) than simple versions of the CDM theory predict. We argue here that the successes of the CDM theory can be retained and the new observations accommodated in a spatially flat cosmology in which as much as 80% of the critical density is provided by a positive cosmological constant, which is dynamically equivalent to endowing the vacuum with a non-zero energy density. In such a universe, expansion was dominated by CDM until a recent epoch, but is now governed by the cosmological constant. As well as explaining large-scale structure, a cosmological constant can account for the lack of fluctuations in the microwave background and the large number of certain kinds of object found at high redshift.

The main assumptions of the CDM cosmogony are that the Universe is flat and dominated by weakly interacting particles, and that gaussian, scale-invariant, adiabatic fluctuations were generated in the early Universe and grew by gravitational instability to form the structure that we observe today. These assumptions are compatible with the inflationary picture of the early Universe⁹, with the observational fact that most of the matter in the Universe is dark¹⁰ and with the standard model of primordial nucleosynthesis¹¹ which indicates that baryons contribute only $\Omega_B \approx 0.016 h^{-2}$ (where Ω is the mean mass density relative to the critical density of the Einstein-de Sitter model, $\rho_c = 3H_0^2/8\pi G$, the subscript B refers to the baryonic component). Conventionally it has been assumed that the cosmological density parameter Ω_0 is unity and that the cosmological constant Λ is zero. But Peebles¹² has pointed out that low-density universes are spatially flat if $\Lambda_0 = 3(1 - \Omega_0)H_0^2$ and so might be compatible with inflation. Here we expand on this suggestion.

In the standard version of the CDM model, the power spectrum of linear density fluctuations retains its primordial scale-invariant form, $P(k) \propto k$, on large scales but changes slope to $P(k) \propto k^{-3}$ on small scales. The transition between these two regimes occurs at a physical scale $\lambda_{\text{equ}} \approx c t_{\text{equ}} \approx 10(\Omega_0 h^2)^{-1}$ Mpc, where t_{equ} is the time at which matter and radiation have equal densities. Detailed calculations¹³ give

$$P(k) \propto k/[1 + (ak + (bk)^{3/2} + (ck)^2)^\nu]^{2/\nu} \quad (1)$$

if $\Omega_B < \Omega_0$, where $a = 6.4(\Omega_0 h^2)^{-1}$ Mpc, $b = 3.0(\Omega_0 h^2)^{-1}$ Mpc, $c = 1.7(\Omega_0 h^2)^{-1}$ Mpc and $\nu = 1.13$. The shape of the power spectrum depends only on the value of $\Omega_0 h^2$ and not on Λ_0 , so we would expect more large-scale structure in a low-density spatially flat CDM universe than in the canonical $\Omega_0 = 1$ model.

Figure 1 shows angular two-point correlation functions $w(\theta)$ for galaxies from the deep APM (named after the Automatic Plate Measuring machine in Cambridge) galaxy survey⁵. The angular correlation function is related to the spatial two-point correlation function $\xi(r)$ by an integral equation¹⁴, and given a model for $\xi(r)$ it is thus straightforward to calculate $w(\theta)$ if one knows the redshift distribution of the galaxy sample. The dotted line in Fig. 1 shows $w(\theta)$ for the 'standard' $\Omega = 1$ CDM model^{3,15} as calculated in ref. 5. The dotted line falls well below the data, showing that the APM galaxy survey contains much more structure on scales $\geq 10 h^{-1}$ Mpc than expected in the standard CDM model. The thin solid line shows $w(\theta)$ for a model with $\Omega_0 = 0.2$, $h = 1$ calculated using the linear-theory power spectrum of equation (1), and the dashed line shows a model with $\Omega_0 = 0.2$,

$h = 0.75$. In each of these cases, the amplitude of the power spectrum has been adjusted so that the variance of the density fluctuations in spheres of radius $8 h^{-1}$ Mpc is unity, $\sigma_8^2 = 1$, as observed of the galaxy distribution¹⁶. The latter two curves bracket the results from the APM galaxy survey on angular scales $> 1^\circ$ (corresponding to physical scales of $\geq 5 h^{-1}$ Mpc) but fall below the observations on smaller scales, where $\xi(r)$ exceeds unity and linear perturbation theory is expected to fail.

To determine the shape of $\xi(r)$ in the nonlinear regime, and also to check the accuracy of the linear theory in the transition region $\xi(r) \approx 1$, we have run 13 N -body simulations of spatially flat low-density CDM universes using the P³M technique¹⁷. Each model contains 32,768 particles in a cubical box; we chose a box of present size $L_b = 50 h^{-2}$ Mpc for three models, $L_b = 150 h^{-2}$ Mpc for five models and $L_b = 300 h^{-2}$ Mpc for the remaining five models. The amplitude of the initial fluctuation spectrum was chosen to give $\sigma_8^2 = 1$ for $h = 1$ and $\Omega_0 = 0.2$ after the universe had expanded by a factor of 3 for the models with $L_b = 150 h^{-2}$ and $300 h^{-2}$ Mpc, and by a factor of 3.8 for the models with $L_b = 50 h^{-2}$ Mpc. Figure 2 shows the spatial two-point correlation function $\xi(r)$ from the N -body simulations at the final epoch. The solid line shows the linear-theory correlation function determined from equation (1) which provides an excellent match to the N -body results on scales $\geq 3 h^{-2}$ Mpc where $\xi(r) \leq 2$. The dotted line shows a power law, $\xi(r) = (r/5.5 \text{ Mpc})^{-1.7}$, similar to the observed two-point galaxy correlation function at small scales. The N -body results clearly rise above the dotted line on small scales. The thick solid line in Fig. 1 shows $w(\theta)$ calculated from the N -body results in Fig. 2 for $h = 0.9$. This curve provides an excellent match to the APM results on large scales, but overshoots on small scales. Evidently, if this model is to match the observations, galaxies cannot be accurate tracers of the mass distribution on scales where the fluctuations are highly nonlinear. We do not regard this as a particularly serious discrepancy because our models neglect physics that is likely to be important on small scales where $\xi(r) \gg 1$. The main conclusion to be drawn from this comparison is that a CDM model with $\Omega_0 h \approx 0.2$ can account for the large-scale clustering of galaxies seen in the APM galaxy survey.

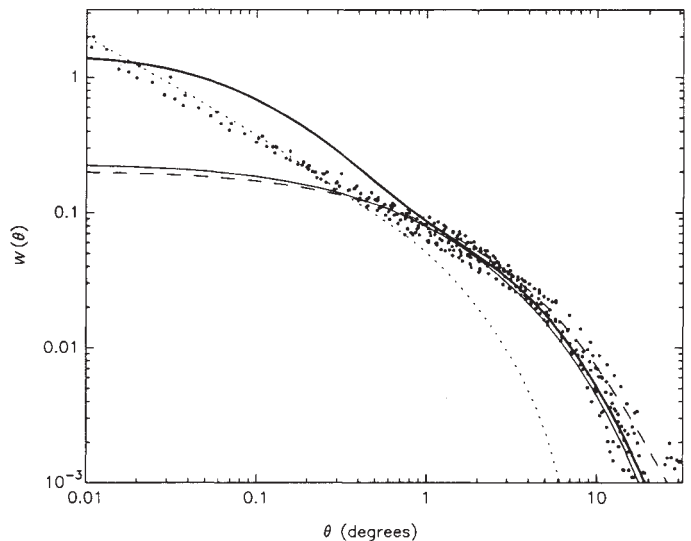


FIG. 1 The dots show estimates of the angular correlation function $w(\theta)$ for galaxies in the APM galaxy survey (see ref. 5 for details). These estimates have been scaled to the depth of the Lick galaxy catalogue where 1° corresponds to a spatial scale of $\sim 5 h^{-1}$ Mpc. The dotted line shows the predictions of the $\Omega = 1$ CDM model (from ref. 5). The thin solid and dashed lines show the results of the linear theory for $\Omega_0 = 0.2$ scale-invariant CDM models with $h = 1$ and 0.75 , respectively. The thick solid line shows N -body results for $\Omega = 0.2$ and $h = 0.9$; the flattening of this curve at angular scales $\leq 0.1^\circ$ is an artefact of the resolution of the computer code, but the excess between 0.1° and 1° is real (see Fig. 2).

TABLE 1 Comparison of observed excess variance $\sigma^2(l)$ with model predictions

l (h^{-1} Mpc)	10	20	30	40	60
IRAS* survey	0.68–1.10	0.31–0.57	0.17–0.38	0.14–0.32	0.017–0.11
CDM† $\Omega=1$ $h=0.5$	1.06	0.35	0.16	0.07	0.02
CDM‡ $\Omega=0.2$ $h=1.0$	1.69	0.58	0.27	0.15	0.06
CDM‡ $\Omega=0.2$ $h=0.75$	1.65	0.62	0.31	0.18	0.09
CDM§ $\Omega=0.2$ $h=0.9$	1.73	0.58	0.30	0.18	0.08

* 95% confidence limits on $\sigma^2(l)$ from ref. 6.

† Calculated as described in ref. 6.

‡ Calculated using linear theory as described in the text.

§ Calculated from the N -body simulations described in the text.

We now examine results on large-scale clustering from a new redshift survey of over 2,000 galaxies detected by IRAS (infrared astronomical satellite). Efstathiou *et al.*⁶ have measured the excess variance above the Poisson level, $\sigma^2(l) = ((\Delta N)^2 - \langle N \rangle) / \langle N \rangle^2$, of the galaxy counts in cubical cells of length l . The 95% ranges of σ^2 determined from the redshift survey for cells of various lengths are listed in Table 1, together with the expected values for the standard $\Omega=1$ CDM model from ref. 6. The observed values of σ^2 exceed the predictions of the standard CDM model indicating a discrepancy similar to that shown in Fig. 1. The variance $\sigma^2(l)$ is related to the power spectrum $P(k)$ by

$$\sigma^2(l) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} P(k) \frac{\sin^2(k_x l/2)}{(k_x l/2)^2} \times \frac{\sin^2(k_y l/2) \sin^2(k_z l/2)}{(k_y l/2)^2 (k_z l/2)^2} d^3 k \quad (2)$$

where we have ignored the distortions in mapping from real space to redshift space¹⁸. The third and fourth rows in Table 1 list values of $\sigma^2(l)$ calculated from equations (1) and (2) for the $\Omega_0=0.2$ CDM models with $h=1$ and $h=0.75$. The fifth row lists σ^2 determined from our N -body simulations in which we have included the distortion of clustering in redshift space. These results show that a low-density CDM model can provide an excellent match to the large-scale clustering of IRAS galaxies in addition to the clustering measured from the APM galaxy survey.

Our analysis shows that a low-density CDM model can account for structure in the Universe on scales $\geq 5h^{-1}$ Mpc in quantitative detail. But are there other plausible explanations and why do we wish to invoke a cosmological constant? It is perhaps possible that matter in the Universe is distributed in a completely different way to galaxies. This would seem unlikely on scales of about 10 – $50h^{-1}$ Mpc, where the density field is presumably linear, and is not supported by comparisons of the dipole anisotropy for optical and IRAS galaxies with our motion with respect to the microwave background radiation¹⁹, or by a comparison of the velocity field predicted from the galaxy distribution in the IRAS survey with observed peculiar velocities²⁰. If fluctuations in the galaxy distribution are indeed proportional to fluctuations in the mass, then our analysis shows that the power spectrum at large scales must resemble equation (1) with $\Omega_0 h \approx 0.2$. The CDM model is attractive because it links the formation of cosmic structure to plausible physics of the early Universe, so it would seem reasonable to retain the basic picture as far as possible. If $\Omega=1$, we might be able to mimic the power spectrum of a low-density CDM model, without any fundamental change in the underlying cosmological model, by postulating departures from a scale-invariant spectrum or by tampering with the matter content of the Universe. The former has been explored by several authors within the context of the quantum generation of fluctuations during inflation (see ref. 21), but requires extreme fine tuning of the parameters. Examples of the latter include CDM universes with a high baryon density, short-lived massive neutrinos²², or one or more flavours of light (~ 1 eV) neutrino (J. R. Primack, personal communication).

We can, however, simply accept that $\Omega_0 \approx 0.2$, while retaining the key ingredients of the CDM model, namely a flat universe with scale-invariant, adiabatic initial fluctuations. This requires a positive cosmological constant and is compatible with inflation¹². Furthermore, spatially flat scale-invariant CDM models with $\Omega_0 h \approx 0.2$ are compatible with limits on the anisotropies of the microwave background radiation²³, whereas equivalent low-density models with $\Lambda=0$ are firmly excluded by these limits¹⁴. There are several other reasons why CDM models with a positive cosmological constant should be taken seriously, some of which clearly favour a model with a change in the underlying geometry rather than just a change in the shape of the power spectrum. (1) Observations of the peculiar motions of galaxies⁷ on large scales suggest higher streaming flows than predicted by the standard CDM model (although the interpretation of these results is controversial, see ref. 24 for a detailed discussion). The extra large-scale fluctuations in low-density CDM models lead to larger streaming motions which are broadly consistent with those observed²⁵. (2) A flat universe with a non-zero Λ has a larger volume out to a given redshift than one with $\Lambda=0$. This may explain the large abundances of certain classes of high-redshift object without requiring dramatic evolution (L. Cowie and S. Lilly, personal communication; ref. 26), for example, the large surface density of faint blue galaxies recently detected²⁷ and the numbers of damped Lyman- α systems seen in the spectra of high-redshift quasars²⁸. (3) Recent determinations of the Hubble constant indicate $h \approx 0.8$ (ref. 29), which is incompatible with estimates of the age of the Universe if $\Omega=1$ but may be compatible with spatially flat low-density models¹². (4) A density parameter of $\Omega_0 \approx 0.2$ is suggested by analyses of the small-scale peculiar motions of galaxies^{16,30}, and thus this model may not require galaxies to be biased strongly relative to the mass (although some bias may be necessary if

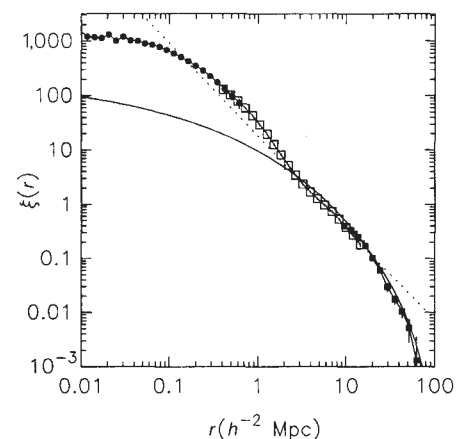


FIG. 2 Two-point correlation functions from N -body simulations of the $\Omega_0=0.2$, spatially flat CDM universes discussed in the text. Filled circles show results from simulations with a box size of $L_b=50h^{-2}$ Mpc, open squares for models with $L_b=150h^{-2}$ Mpc and filled squares for models with $L_b=300h^{-2}$ Mpc. The solid line shows the predictions of linear theory and the dotted line shows the power law $\xi(r)=(r/5.5 \text{ Mpc})^{-1.7}$. The shallow slope at $r \leq 0.1h^{-2}$ Mpc results from the finite resolution of the N -body code.

dynamical friction lowers the peculiar velocities of galaxies relative to the mass, as suggested by recent numerical experiments³¹). (5) White (personal communication) has pointed out the following argument in favour of a low-density universe. Within $1.0h^{-1}$ Mpc of the centre of the Coma cluster, the observed mass in stars and gas is a fraction of $\sim 0.06 + 0.046 h^{-1.5}$ of the total mass³². This is presumably a lower limit on the baryon fraction of the cluster, whereas the upper limit on the baryon fraction of the Universe may be as small as $\Omega_B/\Omega_0 < 0.016/(\Omega_0 h^2)$ (ref. 11). For $h \geq 0.5$, agreement between these two limits requires $\Omega_0 \leq 0.3$, in line with the parameters required to reproduce the observed large-scale structure. (6) Galaxy formation occurs at a higher redshift in a flat low-density CDM universe than in one with $\Omega = 1$, which some authors³³ would consider an attractive feature.

A non-zero cosmological constant would have profound implications for fundamental physics³⁴. Although there have been several recent attempts to explain why the cosmological constant should be zero, most notably by Coleman³⁵, it is possible that the cosmological constant at the present time is dynamically important much in the same way that the vacuum energy dominates in models of inflation, although this would require extraordinary fine tuning of physical parameters (see ref. 36 for example). Nevertheless, a positive cosmological constant could solve many of the problems of the standard CDM model and should be taken seriously. The detection of anisotropies in the microwave background would seem to offer the best test of the low-density CDM models discussed here, although other tests, such as geometric tests of the deceleration parameter q_0 and gravitational lensing of quasars³⁷ and distant galaxies, may be capable of setting interesting constraints. $\square$

On the fast recovery of the Vela pulsar from its Christmas 1988 glitch

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THE Vela pulsar, whose most recent (eighth) glitch occurred during an observing session¹, is the first to have been 'caught in the act'. Post-glitch timing observations² of the rapid (≤ 1 day) recovery of the pulsar's rotation put significant constraints on the conventional model of a glitch, according to which vortices in the rotating superfluid interior of the neutron star, whose ends are pinned to nuclei in the star's crust, abruptly move to new pinned locations³. The large initial jump in the spin-down rate $\dot{\Omega}$ and its prompt recovery seem to imply that an improbably large 20% of the total moment of inertia resides in the crust. We show here, however, that the fast response can be understood in terms of the dynamics of a portion of the pinned crustal superfluid^{4,5}, amounting to $\sim 5.4 \times 10^{-3}$ of the total moment of inertia, linearly coupled to the star's superfluid interior. This fraction is consistent with values previously deduced for the Vela pulsar⁶ and others, including PSR0355+54 (ref. 4), which also showed a large jump⁷ in $\dot{\Omega}$. By modelling the post-glitch relaxation, we estimate the vortex pinning energy at ~ 0.3 MeV, also consistent with earlier estimates.

The equations of motion for the neutron-star crust, rotating at angular velocity Ω_c , coupled to a component of the pinned crustal neutron superfluid which responds linearly to changes in Ω_c may be written in the form

$$I_c \dot{\Omega}_c = N_{\text{ext}} - I_p \dot{\Omega}_s \quad (1a)$$

$$\dot{\Omega}_s = -\frac{(\Omega_s - \Omega_c)}{\tau} \quad (1b)$$

Here N_{ext} is the external magnetospheric torque that acts to spin down the pulsar. I_c is the moment of inertia of the crust and the quantum liquid core, which is expected to couple to the crust on very short timescales^{8,9} and comprises almost all of the stellar moment of inertia I , and I_p ($\sim 10^{-3}$ – $10^{-2} I$) is the moment of inertia of the portion of the pinned crustal superfluid, rotating at the rate Ω_s , that is coupled linearly to the crust with a characteristic time τ . Equations (1a) and (1b) which were used in the initial two-component model of neutron-star dynamics¹⁰, are applicable in any model in which the response of the superfluid to changes in Ω_c is linear, and hence also describe the linear response of vortex creep. Solving these equations for the superfluid response to an observed glitch, $\Delta\Omega_c$, we obtain

$$\Delta\dot{\Omega}_c(t) = \frac{-I_p}{I} \frac{\Delta\Omega_c(0)}{\tau} e^{-t/\tau} \quad (2a)$$

$$\left(\frac{\Delta\dot{\Omega}_c}{\dot{\Omega}_c}\right)_0 = \frac{2I_p}{I} \frac{\Delta\Omega_c(0)}{\Omega_c} \frac{t_{\text{sd}}}{\tau} \quad (2b)$$

where $(\Delta\dot{\Omega}_c)_0$ is the initial jump in $\dot{\Omega}_c$ at the time of the glitch, $I = I_p + I_c$, and we have introduced the pulsar spin-down time

$$t_{\text{sd}} \equiv \frac{\Omega_c}{2|\dot{\Omega}_c|} \quad (3)$$

Applying equation (2b) to the fastest exponentially relaxing components of post-glitch behaviour identified by Flanagan² and by McCulloch and Hamilton¹¹ gives the values of I_p/I

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shown in Table 1. The results of the data analysis of these observations agree qualitatively, but differ in detail because at longer timescales the multicomponent relaxation can be fitted with somewhat different combinations of parameters², and because the shortest relaxation times that can be resolved depend on the data span used in the fits (ref. 2, and P. M. McCulloch and P. A. Hamilton, personal communication). The analysis of 12-hour data spans starting 35 minutes after the glitch¹² disclosed the component of the post-glitch response that relaxes exponentially with a time constant of 0.4 day. This component had not been observed previously^{11,13-15}, because earlier post-glitch observations started, at best, eight hours after the actual glitch^{14,15}.

Our conclusion, that $I_p/I \ll (\Delta\dot{\Omega}_c/\dot{\Omega}_c)_0$, is at first sight surprising, but it is a consequence of the fact that the magnitude of the glitch

$$\Delta\Omega_c \approx 1.3 \times 10^{-4} \text{ rad s}^{-1} \quad (4a)$$

is large compared to the steady-state lag

$$(\Omega_s - \Omega_c)_\infty = |\dot{\Omega}|_\infty \tau \approx 3.5 \times 10^{-6} \text{ rad s}^{-1} \quad (4b)$$

obtained from equations (1a) and (1b) using the condition that in steady state both the superfluid and the crust spin down at the same rate, $\dot{\Omega}_s = \dot{\Omega}_c = \dot{\Omega}_\infty = N_{\text{ex}}/I$. Thus from a steady-state situation before the glitch, with the superfluid rotating slightly faster than the crust, we jump to a post-glitch situation with the crust rotating faster, by much more than the steady-state lag. Immediately after the glitch, the superfluid must actually spin up for a while to approach Ω_c and correct the post-glitch $\Omega_s - \Omega_c$, which has changed sign and acquired a magnitude $\Delta\Omega_c$ large compared to steady-state conditions. The external torque has not changed, but the superfluid is temporarily spinning up. Under these conditions the crust has a $\Delta\dot{\Omega}_c/\dot{\Omega}_c$ that is larger than the moment-of-inertia ratio I_p/I . We emphasize, as a model-independent statement, that any linear-response model of the dynamical coupling that has the form of equations (1a) and (1b) can give a value of $\Delta\dot{\Omega}_c/\dot{\Omega}_c$ that is large compared to the moment-of-inertia fraction of the relaxing component if $\Delta\Omega_c$ is large enough (see equation (2b)).

The most remarkable, and in our view the most telling, property of post-glitch relaxation is that all components, whether responsible for linear response (simple exponentials) or persistent or slowly recovering shifts in $\dot{\Omega}_c$, from Vela and from other pulsars, involve fractional moments of inertia of the order of 10^{-3} – 10^{-2} which is the fractional moment of inertia of the neutron-star crust superfluid.

We next use the observed 0.4-day relaxation time to deduce the corresponding pinning energy in the vortex-creep model of neutron-star dynamics. According to this model the crust superfluid couples to the spin-down of the star through thermally activated creep of vortex lines, whose motion is hindered by pinning to the crust lattice¹⁶. In the linear-response regime of vortex-creep theory³, the pinning energy in the regions with relaxation time τ is

$$E_p = kT \left[21.1 + \ln \tau + \ln \left(\frac{b_{-12} \xi_{-12} \rho_{14} v_{0.7}}{kT/10 \text{ keV}} \right) + \ln \left(\frac{\Omega}{\Omega_{\text{Vela}}} \right) \right] \quad (5)$$

Here ξ_{-12} is the superfluid coherence length in units of 10^{-12} cm, b_{-12} is the distance between pinning sites along a vortex line (in the same units), ρ_{14} is the crustal superfluid density in units of $10^{14} \text{ g cm}^{-3}$, and $v_{0.7}$ is a microscopic vortex velocity in units of 10^7 cm s^{-1} . We take the argument of the last logarithm to be 50–1,000, for a conservative upper bound on E_p . The value of E_p/kT that corresponds to $\tau = 0.4$ day is then between 24 and 27. Using the observational upper limit on the surface temperature¹⁷, together with the canonical surface-interior tem-

TABLE 1 Fitting parameters for the eighth Vela glitch

	Ref. 2	Ref. 11
$\Delta\Omega_c(0)/\Omega_c$	1.8×10^{-6}	1.8×10^{-6}
$(\Delta\dot{\Omega}_c/\dot{\Omega}_c)_0$	2.0×10^{-1}	4.4×10^{-2}
τ (d)	0.4	1
I_p/I	5.3×10^{-3}	3.0×10^{-3}
E_p (MeV)	0.265–0.3	0.275–0.31

The first three rows give the parameters of the fastest post-glitch relaxation after the eighth Vela pulsar glitch^{2,11}. The last two rows give our derived values of the moment-of-inertia fraction and pinning energy in the superfluid regions of the star responsible for the promptest relaxation.

perature relation¹⁸ to obtain $kT \leq 11 \text{ keV}$, we find that $E_p \approx 0.265$ – 0.3 MeV . These values of the pinning energy are consistent with the criteria of the linear creep regime⁵ and reasonable for estimates with a specific model of the pinning mechanism⁵. As shown in Table 1, similar pinning energies are obtained from the 1-day relaxation time fitted by McCulloch and Hamilton¹. A detailed discussion of these issues and an evaluation of all Vela post-glitch timing observations to date within the framework of vortex-creep theory will be presented elsewhere (M.A.A., H. F. Chen, K. S. C. and D. P., manuscript in preparation). $\square$

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Lensless imaging due to back-scattering

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WE demonstrate that light scattered from a disordered medium or a roughened surface will form an image of the light source without the use of any focusing optics. The image is superimposed on a relatively uniform background of scattered light, which we are able to remove by suitably processing the output from a CCD (charge-coupled device) detector. Images of several sources have been obtained, using both laser and incandescent light sources. We interpret the imaging as being caused by coherent back-scattering, a phenomenon which has been invoked to explain a well-known intensity maximum in the far-field back-scattered light from a random medium¹⁻⁹. Our work amplifies and explains an earlier report¹⁰ of 'pseudo-imaging' of a point source by 'retro-reflection'.

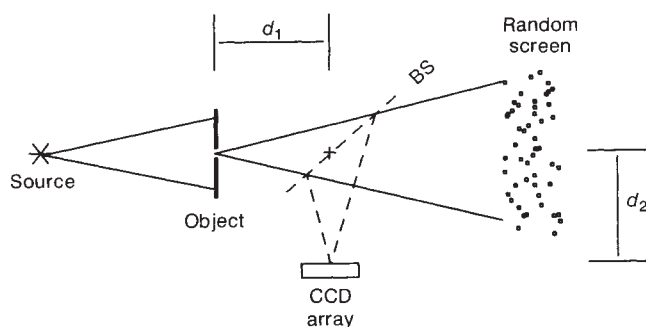


FIG. 1 Experimental configuration for the observation of image due to back-scattering. The image is at best focus when $d_1 \approx d_2$. BS, beam splitter.

Figure 1 shows a diagram of the experimental set-up, which we have kept as simple as possible to avoid misinterpretation of the observed images. A light source illuminates a slide which acts as the object to be imaged. We have used several sources including HeNe and Ar lasers, as well as a tungsten lamp. The diverging light emerging from the slide passes through a 50%-transmission beam splitter and falls on a rough screen from which the light is randomly scattered. The screen consists of a suspension of $0.3\text{-}\mu\text{m}$ polystyrene spheres, at a concentration of 10^{11} cm^{-3} . We have also used latex paint diluted 1 to 10 in water, and non-glossy paper. The light that is scattered back towards the slide is intercepted by the beam splitter which redirects it towards the detector, a 512×256 CCD array with pixel dimensions $23 \times 23\text{ }\mu\text{m}$. We have used $d_1 \approx d_2 \approx 20\text{ cm}$, and the distance from the beam splitter to the screen could be varied from 10 to 30 cm without substantially affecting the image.

We find an image of the object on the slide superimposed on a homogeneous background, the brighter points of the image being $\sim 10\%$ brighter than the background. The data obtained from the CCD array can be processed to remove the background and enhance the remaining image. Figure 2 shows the enhanced image of a letter *L* obtained in this fashion. The original letter was a simple *L* with dimensions $5 \times 3\text{ mm}$ and line width 0.8 mm . The illuminating source was a tungsten lamp and the screen was a sheet of white paper. As can be seen, the image obtained from the back-scattering is reasonably faithful but is somewhat lacking in resolution. Nevertheless, it demonstrates quite clearly the imaging effect due to back-scattering from a disordered medium.

To explain the imaging properties of a random scattering medium, we offer the following simple model which is based on similar models used to interpret coherent back-scattering¹⁻⁹. Consider the situation depicted in Fig. 3. We concentrate on

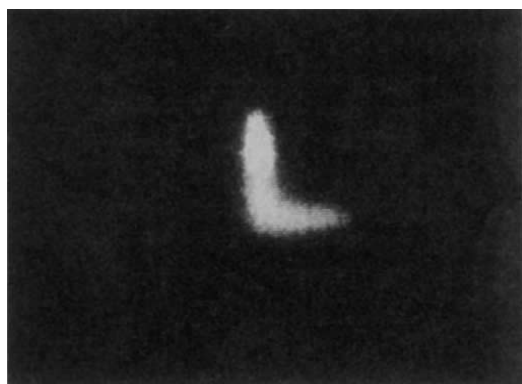


FIG. 2 The processed image of a letter *L* as registered on the CCD array. Much of the homogeneous background has been removed.

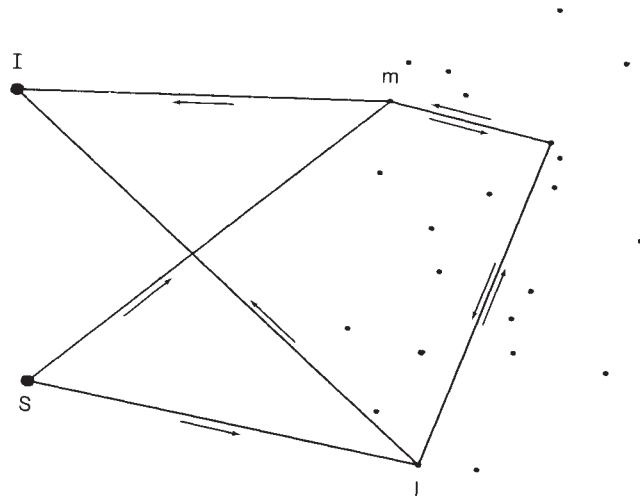


FIG. 3 A possible multiple-scattering configuration: S, source; I, image point; l and m are scattering centres.

two particles *l* and *m* on the scattering screen. Light is emitted from a source *S* and is first scattered by particle *l*, randomly scattered in the medium, and finally scattered towards the imaging point *I* by particle *m*. There is an alternative path in which the light is first scattered by particle *m*, follows the same random scattering path as before but in reverse, and is finally scattered towards the imaging point by particle *l*. The net phase difference ϕ between these paths is

$$\phi = \frac{2\pi}{\lambda} (|\mathbf{r}_1 - \mathbf{r}_S| + |\mathbf{r}_m - \mathbf{r}_l| - |\mathbf{r}_m - \mathbf{r}_S| - |\mathbf{r}_1 - \mathbf{r}_I|) \quad (1)$$

where λ is the wavelength, and $\mathbf{r}_S$, $\mathbf{r}_I$, $\mathbf{r}_l$ and $\mathbf{r}_m$ are the positions of the source, image, particle *l* and particle *m*, respectively. This phase difference leads to an interference term when the two waves are added at the imaging point such that when the object and imaging points are sufficiently far from the two scattering points *l* and *m*, the irradiance at the image point due to a particular random path *n*, can be shown to be given by

$$I(\mathbf{r}_I, \mathbf{r}_m, \mathbf{r}_S, \mathbf{r}_l, n, \lambda) = C(\mathbf{r}_I, \mathbf{r}_m, \mathbf{r}_S, \mathbf{r}_l, n, \lambda) \times \left[1 + \cos\left(\frac{2\pi}{\lambda z} (\mathbf{r}_I - \mathbf{r}_m) \cdot (\mathbf{r}_S - \mathbf{r}_l) \right) \right] \quad (2)$$

where *z* is the distance from the source to the screen, and the function *C* is a smooth function of the parameters and describes the probability of the particular scattering event. The second term leads to the imaging effect because it contains an interference term. For a random medium and a broadband source, the average of the cosine term will be zero unless $\mathbf{r}_S \approx \mathbf{r}_I$; at $\mathbf{r}_S \approx \mathbf{r}_I$ the irradiance for that particular scattering event is doubled.

We note that the image resolution is controlled by the average interparticle distance $|\mathbf{r}_l - \mathbf{r}_m|$ for which the probability function *C* is not zero. If this distance is small then the interference term can be significant over a wider range of the distance $|\mathbf{r}_S - \mathbf{r}_I|$. The dot product in the interference term also increases the range for the observation of the interference, which explains why we can still observe the image at 2 mm from the 'focus' position. We conclude that the irradiance at any imaging point is the sum of all possible scattering events, but that when the image point is at the source point, there is an increase in the irradiance due to constructive interference between time-reversed scattering paths. (Single scattering events do not have this interference term). Because all the source points on an extended source will undergo this enhanced back-scattering, an image of the source will be produced by multiple scattering. The image produced

can be observed by intercepting the back-scattered light as we have done here. □

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Radiocarbon dating of prehistoric rock paintings by selective oxidation of organic carbon

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DATING of prehistoric rock paintings (pictographs) has traditionally relied on indirect evidence. This includes inferences based on the archaeological context, such as superpositions of pictorial styles¹ and the depiction of images that constrain their ages^{1,2}, as well as dating of deposits that either cover the art *in situ*^{3,4} or contain separated fragments of the painted surface⁵. Migration of ions between the bulk rock and the natural coatings that form on a newly exposed surface has also been exploited to date petroglyphs (rock carvings) in desert regions^{6–13}. Until recently^{14–17}, however, direct dating (by radiocarbon techniques) of pictographs has not been possible^{18,19}, mainly because of the problem of separating inorganic carbon from the organic material in the pigments. Here we report on a new technique which allows this separation to be effected by using a low-temperature, low-pressure oxygen plasma to oxidize selectively the organic component; this may then be analysed using standard ¹⁴C methods. We have applied this technique to a portion of a pictograph from the Lower Pecos region of southwest Texas (Fig. 1). The date obtained, 3,865 ± 100 yr BP (before present) is consistent with that expected on the basis of archaeological inference²⁰. As organic carbon is a ubiquitous component of pictograph paints, this technique should be applicable to rock paintings throughout the world.

Direct radiocarbon dating of prehistoric rock paintings requires that organic material was incorporated into the paints, and that this carbonaceous matter can be extracted without contamination from other sources. The paint pigments themselves are inorganic, primarily iron and manganese oxides²¹. These materials do not adhere to limestone surfaces, however, so a binding medium was used to attach the pigments to the substrate. Chemical and spectrographic analyses of prehistoric pictograph paints used in the Four Corners region of Utah, New Mexico, Colorado and Arizona show that a variety of readily available organic materials were used as binders, including blood, eggs, seed oils, plant resins and juices, milk, honey, animal oils and urine¹⁹. This variety of organic materials led us to think it likely that similar materials were used in the Lower Pecos region. We were, however, unable to characterize the organic binding material in our small paint sample. As our technique does not require a specific organic component, it is more generally applicable than earlier dating of cave paintings by ¹⁴C acceleration mass spectrometry (AMS)^{14–17}.

It is our use of a radiofrequency-generated oxygen plasma that allows the separation of the organic matter used in the paint from the limestone (CaCO₃) substrate. This low-temperature (~100 °C), low-pressure (~4 torr) oxygen plasma has a catalytic effect in oxidizing virtually all types of carbonaceous materials^{22–24}. In the otherwise mild conditions, the fully oxidized carbonate rock is unaffected²⁵. The plasma extraction system consists of a glass high-vacuum chamber between two external copper electrodes connected to a 13.56-MHz radio-frequency generator. Only glass or metal surfaces are subjected to the plasma. A rotary pump provides an initial rough vacuum; this is separated from a liquid-nitrogen sorption pump by a valve and a room-temperature molecular-sieve oil trap to avoid contamination of the system with pump oil. We eliminate atmospheric CO₂ contamination by evacuating the reaction chamber to a pressure of ~10⁻⁷ torr using the sorption pump and an ion pump before introducing pure oxygen. Calculations show that a pressure of 2.5 × 10⁻⁵ torr is sufficiently low to ensure that atmospheric CO₂ would contribute <1 part per 10⁹ (p.p.b.) to the carbon content of the paint sample. The chamber was thoroughly cleansed of organic material by several cycles in which the oxygen plasma was operated for several hours (total time 18 h) and then the system pumped down. The oxygen plasma oxidized all organic contamination in the chamber to CO₂ and H₂O; continuous heating of all surfaces (180 °C) and subsequent vacuum pumping prevented adsorption of the gases onto the glass surfaces.

After introducing the sample into the system, we maintained a glow discharge of ~4 torr of oxygen for a week to ensure that all organic material in the paint was converted to CO₂ and H₂O. The products were collected in an extraction finger by freezing with liquid nitrogen and sent to Beta Analytic Inc. There, after the water was removed, the CO₂ was purified and reacted with hydrogen on a cobalt catalyst producing 12 mg of graphite. The ¹⁴C content of this graphite was determined by AMS at Eidgenössische Technische Hochschule (ETH) in Zurich. (Beta Analytic and ETH numbers of our sample are Beta-33586 and ETH-5909.)

Triplicate AMS measurements yielded an age of 3,865 ± 100 BP. The date from the ETH ¹⁴C measurement was calculated by international convention as radiocarbon years before AD 1950; the half-life of ¹⁴C was taken to be 5,568 yr and 95% of the activity of the National Bureau of Standards oxalic acid (original batch) was used as the modern standard. The quoted errors are solely from the counting statistics of the modern standard, background and sample. The variation between the

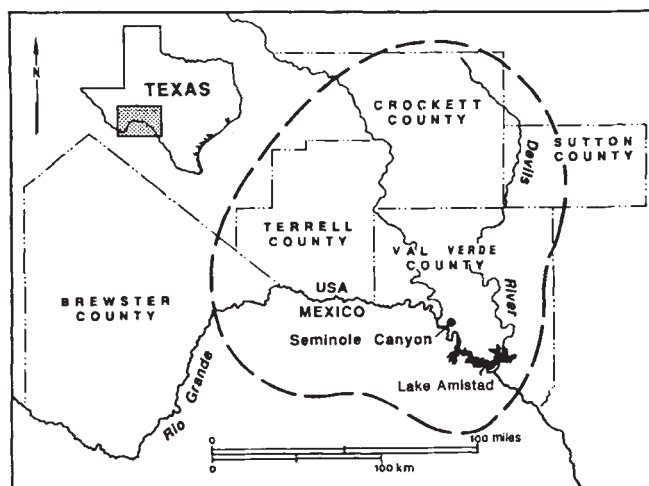


FIG. 1 Map showing the approximate extent of the Lower Pecos region. The sample dated in this study was a naturally exfoliated fragment collected from the floor of rock shelter 41VV75 in Seminole Canyon by one of us (H.J.S.).

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three sample measurements was 0.45%, much lower than the counting errors. This date has not been corrected for the DeVries effect²⁶ or the reservoir effect²⁷, but we have adjusted it for the total isotope effect due to physical and chemical procedures in our laboratory, at Beta Analytic and at ETH. The ^{13}C content ($\delta^{13}\text{C} = [(^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}}] - 1 = 14.5\%$), where the standard is NBS oxalic acid, $\delta^{13}\text{C} = -19\%$, was measured concurrently with that of ^{14}C and ^{12}C in the accelerator beam, allowing for a precise fractionation correction. The proximity of the age obtained with that suggested by dates given for the initiation of the Pecos River style, 4,100–3,200 yr BP²⁰, demonstrates the feasibility of the technique.

The adsorption of carbonaceous matter onto weathering surfaces as they develop into desert varnish, before and after paint application, is a possible source of minor contamination. A previous radiocarbon dating study of desert varnish on lava flows of known age²⁸ suggests a contamination level of <5% in our measurement if the desert varnish on limestone from the Lower Pecos region incorporates the same fraction of organic material as it does on a lava flow from southern California.

There is also the possibility that a small amount of limestone carbon could be released as carbon dioxide. Limestone (CaCO_3 , calcite) decomposes on heating to yield calcium oxide and carbon dioxide, but temperatures in excess of 600 °C are necessary for a noticeable decomposition rate²⁹. No change was found in the calcite present in high-volatile-content bituminous coal after plasma oxidation at reaction temperatures <190 °C, as evidenced by X-ray diffraction²⁵ (which can detect decomposition of calcite to calcium oxide at a level of a few per cent or less). Flow-through oxygen was used in that study, which in our experience is more vigorous than our static operation. Because the limestone in our plasma reaches only ~100 °C, it is unlikely that carbon dioxide from the limestone is a contaminant.

The specificity of the oxygen plasma in oxidizing organic carbon to CO_2 at low temperatures is generally applicable to all materials previously dated by ^{14}C . It would be particularly useful in those cases where the samples to be dated are contaminated with carbonate. Other possible uses of the technique would be on unfired painted pottery, such as that found at some archaeological sites in New Mexico, and on smudged pots found at archaeological sites globally. In these cases, the entire artefact could be placed into the reaction chamber. The method leaves the sample fabric physically intact, but may change the colours of the paint. The effect would be most extreme for black pigments consisting of magnetite (or other iron-containing spinels), which would become red, and for the black carbon-based pigment, which would be removed entirely. For a pot that is black due to magnetite and other metal oxides, the black colour could be restored in a reducing plasma³⁰. Although the tint of red pigments may be slightly changed, it is most likely that a red colour would remain after dating. We could not discern any colour change after dating our pale pink pictograph sample.

Our technique for extracting the organic carbon from rock paintings without contamination from the limestone substrate is applicable to previously undatable rock paintings throughout the world. These graphics had an important role in prehistoric societies, but without chronometric ages, assigning these artefacts to specific cultures is tentative. We attach little significance to our single measurement except as a demonstration of the technique for dating pictographs, irrespective of the organic material used. We are now developing the technique to make it capable of providing accurate and reliable ages, which will aid in the resolution of some of the questions about the chronology of prehistoric rock art. □

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Increases in terrestrial carbon storage from the Last Glacial Maximum to the present

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EVIDENCE from ice cores¹ indicates that concentrations of atmospheric carbon dioxide were lower by about 75 p.p.m. during the Last Glacial Maximum (LGM; ~18,000 years ago) than during the present interglacial (10,000 years ago to the present). The causes of such large changes in atmospheric CO_2 remain uncertain. Using a climate model, Prentice and Fung² have estimated that there was approximately the same amount of carbon in vegetation and soils during the LGM as there was during the present (pre-industrial) interglacial. In contrast, we present here results based on palynological, pedological and sedimentological evidence which indicate that in fact the amount of carbon in vegetation, soils and peatlands may have been smaller during the LGM by $\sim 1.3 \times 10^{12}$ tonnes. Thus, organic carbon in vegetation and soils has more than doubled (from 0.96 to 2.3×10^{12} tonnes) since the LGM. Oceanic CO_2 reservoirs seem to be the only possible source of this large quantity of carbon that has entered the terrestrial biosphere since the LGM (in addition to that which has entered the atmosphere to give the higher interglacial CO_2 levels).

We used published palynological, pedological and sedimentological data to produce global maps of vegetation and ice cover at 18 kyr, which we compare with present natural vegetation (the vegetation types which it is thought would exist without human influence, under present climate conditions). Table 1 gives the areas of each main vegetation type in the approximate categories used by Olson *et al.*³. These results show that drier vegetation types such as desert and arid scrub were much more

extensive around 18 kyr ago than they are now, and that many moist-climate vegetation types were almost absent from large areas that they occupy today (Fig. 1).

Although the definitions of vegetation types we used are not identical to those in the modified Holdridge system used by Prentice and Fung², a few general comparisons are possible. Basing their results on a general circulation model⁴ (GCM), they found that the driest vegetation types would have covered much less area (for example, a 90% reduction in the global area of deserts). The more direct nature of the evidence on which our reconstructions are based leads us to suggest that the GCM overestimated the amount of moisture available to land vegetation at the LGM. Under the lower ambient CO₂ level at the LGM (200 p.p.m., compared with around 270–280 p.p.m. during the present interglacial before anthropogenic forcing began), the efficiency of water use by vegetation may have been reduced⁵. This might have resulted in different relationships between vege-

tation and climate, compared with those during the interglacial period. It is unlikely that this direct CO₂ effect could account for all of the discrepancy between GCM results and palaeo-evidence, however, so it seems that there must have been a considerable reduction in precipitation over much of the land surface.

In general, the vegetation types that prevailed under the cooler drier climates of the LGM would have had a low carbon storage per unit area (Table 2). Even allowing for the expansion of vegetation onto continental shelves exposed by the low sea level during the glacial period, it seems that there would have been far less carbon storage on land at that time. Our calculations, based on present-day measurements of carbon in vegetation and soils, indicate that there would have been an overall increase of around 1,071 Gt (1 Gt = 10⁹ t) in terrestrial carbon storage between the LGM and present natural conditions (Fig. 2). This represents an increase of 110%. The total carbon in vegetation

FIG. 1 The distribution of main vegetation types for *a*, present natural and *b*, the Last Glacial Maximum (18 kyr ago). For clarity, some of the vegetation categories referred to in Table 1 have been grouped together, and these are referred to in brackets in the key. The LGM vegetation map is annotated with the principal literature sources used in constructing the map, numbered according to position in reference list. *a*, Tropical moist forests (equatorial rain forest and seasonal evergreen forest). *b*, Tropical scrub and woodland (tropical woodland, tropical thorn scrub). *c*, Grasslands (tropical grasslands, temperate grasslands, montane grasslands, savanna). *d*, Semi-desert and dry steppe (tropical semi-desert, temperate semi-desert, dry semi-desert steppe). *e*, Temperate broad-leaved forest (warm temperate evergreen, cool temperate deciduous, mediterranean woodland). *f*, Boreal and other closed conifer forest (main taiga, southern continental taiga, conifer rainforest). *g*, Open conifer woodland (northern and dry taiga, open conifer forest, wooded tundra). *h*, Tundra (tundra, boreal peatlands). *i*, Polar desert (northern steppe-tundra, polar desert). *j*, Ice-sheets (ice). *k*, Desert (tropical desert, temperate desert). *l*, Southern steppe-tundra (southern steppe-tundra).



TABLE 1 Global areas and carbon storage for vegetation types

	18 kyr	Present natural	18 kyr	Present natural
	Area (10 ⁶ km ²)	Area (10 ⁶ km ²)	Total vegetation and soil C storage (Gt)	Total vegetation and soil C storage (Gt)
Tropical equatorial forest	1.5	4.7	64.5	202.1
Tropical evergreen seasonal forest	4.9	9.6	127.4	249.6
Tropical woodland (dry open forest)	6.5	11.8	87.7	159.3
Thorn scrub and scrub woodland	5.5	8.0	55.0	80.0
Tropical grasslands	15.6	9.0	51.5	29.7
Savanna	12.4	8.4	92.6	74.7
Temperate and montane grasslands	5.9	10.5	82.6	147.0
Tropical semi-desert scrub	10.5	5.7	25.2	13.7
Temperate semi-desert and dry steppe	5.4	5.0	35.6	33.0
Tropical desert	19.4	12.1	6.4	3.6
Temperate and montane desert	10.1	5.1	31.3	15.8
Mediterranean scrub and woodland	0.7	1.2	10.5	18.0
Temperate broad-leaved deciduous and mixed	1.5	11.4	45.5	330.6
Temperate broad-leaved evergreen forest	1.2	4.3	39.6	141.9
Conifer rainforest	—	0.3	—	19.7
Southern continental taiga	—	3.3	—	92.4
Main taiga	1.4	7.0	32.3	161.7
Northern and maritime open taiga	1.9	5.4	25.8	73.4
Dry open pine woodland	0.4	0.8	7.0	14.1
Wooded tundra and timber line	3.5	0.9	42.0	10.8
Tundra	—	7.4	—	167.9
Northern steppe-tundra and polar desert	13.9	0.7	2.8	<0.1
Southern steppe-tundra	9.7	—	102.8	—
Permanent ice	33.4	14.6	0	0
Sub-total for soils and vegetation	165.3	147.2	968.1	2,039
Wetlands and peatlands	—	2.8	—	280.4
Total	165.3	150.1	968.1	2,319.4

In the absence of direct climate data, it is not possible to classify past vegetation types according to a rigid set of definitions such as the UNESCO system¹² used by Prentice and Fung². Instead, the approximate categories mapped by Olson *et al.*³ are also used as a general description of the structure and composition of both present natural and LGM vegetation. The areas of vegetation types for the present natural situation vary from those given by Olson *et al.*³ because we do not include anthropogenic vegetation types. Where the vegetation has been disturbed by man, we have attempted to reconstruct the type of vegetation that would exist under pre-neolithic conditions. Some vegetation categories used by Olson *et al.* have been 'lumped' together to simplify the calculation. Some vegetation types that exist today do not appear to have been present at the LGM over areas large enough to map. Two categories, northern and southern 'steppe-tundra', have been created to accommodate the open vegetation types with no present analogue but which were widespread in the Northern Hemisphere at around the LGM. The LGM reconstructions assume a 130-m drop in sea level as the boundary for land vegetation, which is at the extreme upper end of the range of estimates for the decrease in sea level during the glacial period. The maps from which our figures are taken are continually being updated and modified as new information is obtained and we hope to publish them in a more detailed form soon. The amount of information available for different regions varies; for some areas the data are still fragmentary, so that only tentative reconstructions are possible.

and soils has probably been greatly depleted during the past two or three thousand years as a result of human activity³—our estimate of present natural vegetation is intended to be a rough representation of the pre-agricultural state during the interglacial. It is also necessary to account correctly for the peat deposits which have accumulated in wetlands during the Holocene⁶. The present peat reserves have been variously estimated at around 202 Gt (ref. 7) or 377 Gt (ref. 8) of carbon. The drier conditions over most of the land surface do not generally seem to have been conducive to peat deposition at the LGM, and the extensive present-day peat basins seem to have begun forming only after the end of the last glacial period (~10,000 years ago)⁶. Using Schlesinger's⁹ estimate for carbon in peatlands as a conservative estimate, and including an estimate for the vegetation on these peatlands, the total increase between the LGM and present natural states is ~1,351 Gt C, a change of 140%. Our carbon storage estimates may not represent the true extremes of the terrestrial carbon storage system during the past 18 kyr. Palynological and other evidence from many

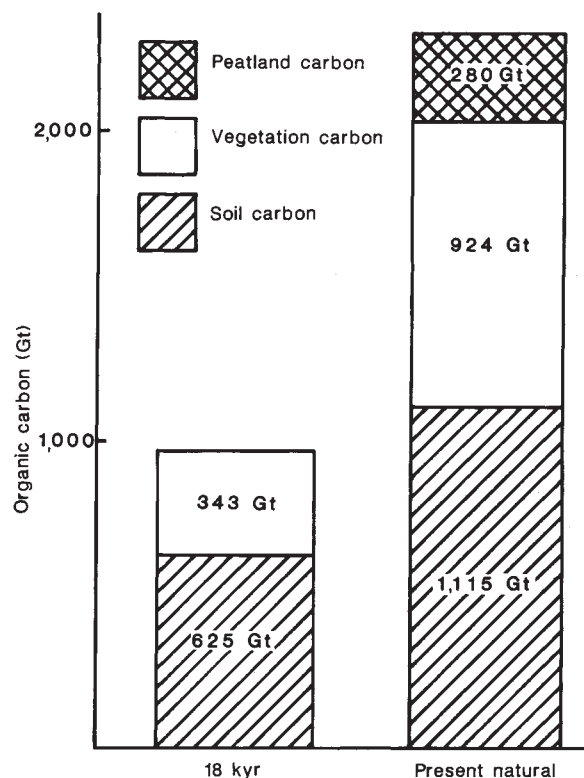


FIG. 2 Comparison of estimated quantities of carbon in soils, vegetation and peatlands/wetlands for 18 kyr and for the present natural state.

TABLE 2 Total carbon storage of vegetation types

	Vegetation C (t ha ⁻¹)	Soil C (t ha ⁻¹)	Total C (t ha ⁻¹)
Tropical equatorial forest	220	210	430
Tropical evergreen seasonal	150	110	260
Tropical woodland (dry open forest)	70	65	135
Thorn scrub and scrub woodland	40	60	100
Tropical grasslands	13	20	33
Temperate and montane grasslands	10	130	140
Savanna	35	54	89
Tropical semi-desert scrub	4	20	24
Temperate semi-desert dry steppe	6	60	66
Tropical desert	1	2	3
Temperate desert	1	30	31
Mediterranean scrub and woodland	70	80	150
Temperate broad-leaved deciduous mixed forest	170	120	290
Temperate broad-leaved evergreen	200	130	330
Coniferous rainforest	400	256	656
Southern continental taiga	140	140	280
Main taiga	115	116	231
Northern and maritime taiga	50	86	136
Open conifer woodland	97	80	177
Wooded tundra and timber line	20	100	120
Tundra	7	220	227
Northern steppe-tundra (glacial)	1	1	2
Southern steppe-tundra (glacial)	6	100	106
Wetlands and peatlands	20	723	743

Carbon storage values (per unit area) for vegetation and soils were derived from various sources^{3,7-9,51-52} and from correspondence and discussion with the authors of these studies. The carbon storage estimates for particular structural and climate vegetation types vary somewhat according to source. We have attempted to assign a set of estimates, to the approximate vegetation categories mapped by Olson *et al.*³, based on consideration of the methods used by each author, and the likely state of vegetation before agricultural disturbance. The northern and southern steppe-tundra have been assigned carbon storage values of present-day polar desert and dry tundra, respectively, on the basis of palynological evidence on vegetation composition and cover (P. A. Colinvaux, personal communication).

parts of the World indicates that moist-climate vegetation was more extensive¹⁰ 9–6 kyr ago, and this probably resulted in increased carbon storage on land.

Our 1,351-Gt increase in terrestrial carbon since the LGM contrasts with the estimate of relatively little change (± 50 Gt) by Prentice and Fung. If all the 'extra' carbon that has entered the terrestrial system since the LGM were to be released into the atmosphere at once, it would raise the atmospheric CO₂ concentration by 635 p.p.m. (if 1 Gt = 0.47 p.p.m. CO₂).

It seems unlikely that much of the carbon that has entered the terrestrial biosphere since the LGM could have been stored in any other form of terrestrial reservoir. There is no good evidence for the sequestering of such vast quantities of organic carbon under ice sheets, or in terrestrial permafrost within the

northern steppe-tundra realm. An oceanic reservoir and 'pump' seems to be the only mechanism by which a sufficiently large quantity of carbon could be added to or removed from the soil-vegetation-atmosphere system¹¹.

Instead of contributing to the lower CO₂ level during the LGM, the terrestrial vegetation and soil carbon reservoirs should be seen as a factor tending to 'damp' the oceanically driven glacial-interglacial CO₂ fluctuation. This is not a true negative feedback effect on atmospheric CO₂, as the size of the terrestrial carbon reservoir would seem to be responding more to climate factors than the level of CO₂ *per se*. □

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***In situ* measurement of interstellar silicon carbide in two CM chondrite meteorites**

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SMALL crystals of silicon carbide, probably of interstellar origin, have previously been isolated from primitive meteorites by dissolution of the host meteorite with acid¹⁻⁸. Here we report the first *in situ* observations of isotopically anomalous SiC^{9,10}. The grains were found by X-ray mapping of polished sections of two chondritic (group CM) meteorites, Cold Bokkeveld and Murchison. Ion microprobe measurements showed ¹³C enrichments, $\delta^{13}\text{C}$, from 199 to 2,800‰, proving that the grains are indigenous. Calculations of the condensation of grains in circumstellar envelopes of different compositions and C/O ratios have suggested that other phases, such as metal carbides, nitrides and sulphides, could form in association with SiC^{11,12}. Etching methods may have destroyed any such phases, had they existed in association with SiC, but our direct detection reveals SiC grains only as isolated matrix particles. This rules out also the possibility that SiC grains were brought into the Solar System as inclusions in larger grains, which protected them from destruction in the solar nebula. Several of the SiC grains we have found are cracked, suggesting that the etching treatment may result in size distributions biased towards smaller grains.

We used the results of previous measurements of SiC in acid residues to define the conditions for the *in situ* search: the concentration is 6–9 p.p.m., most of the mass is in grains <1 μm in size, the background of other silicon-bearing compounds is high, and it is not known whether the grains are randomly distributed. Contamination by terrestrial SiC may be a problem. In addition, polished sections must be prepared without 'plucking' small hard SiC grains from a relatively soft meteorite.

We located the SiC grains in carbon-coated (100 Å) polished sections using a multi-element X-ray mapping method¹³ employing a JEOL 840A scanning electron microscope equipped with a Tracor-Northern Si-Li Micro ZII detector and a 5400 series II image-processing system. The image-processing system was used to display only those pixels that exceeded a threshold value for Si counts. After locating regions corresponding to candidate grains with the SEM, we re-mapped the Si at higher magnification. These enlarged X-ray images usually led to the identification of a specific grain. An ultrathin-window detector was then used to identify SiC by requiring that the grain show C and Si X-ray intensities in the correct ratio with no other elemental peaks present. We then took high-magnification (5,000 $\times$) stereo SEM images.

After locating a number of grains, we treated the section in a commercial oxygen asher to remove the carbon coating. It was then recoated with a thin layer of gold and transferred to a modified IMS-3F Cameca ion probe. The measurements were made with a Cs-ion primary beam using data acquisition and analysis methods similar to those previously used to study SiC in acid residues^{5,14}.

Indigenous grains of SiC were first successfully located in a polished section of the CM meteorite Cold Bokkeveld (CB-4). The section, which was provided by R. Hutchison of the British Museum (Natural History), has been prepared without using SiC abrasives. We made 953 maps at a magnification of 400 and 543 maps at a magnification of 800, giving a total area scanned of 0.503 cm². Of the 38 grains identified, 12 were classified as 'possible contaminants' based on their appearance in SEM stereo pairs. Most of the remaining grains appeared indigenous, but only 8 were large enough for their isotopic compositions to

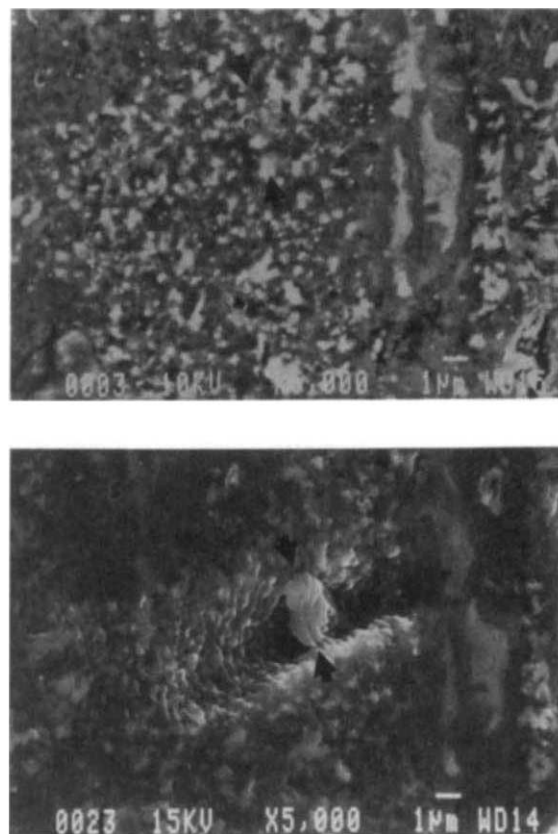


FIG. 1 A buried SiC grain (CB-4-671) in Cold Bokkeveld. *a*, Although a large, bright area (between arrows) was evident in the initial Si X-ray maps, detailed examination of the region failed to show a distinct grain. *b*, Sputtering of the overlying matrix material during ion-probe analysis revealed the previously buried SiC. The carbon isotopic composition was anomalous ($\delta^{13}\text{C} = 440 \pm 7\text{‰}$) confirming that the grain is indigenous. The apparent foliation seen in the analysed grain is probably an artefact introduced by sputtering.

be measured. A further four SiC grains were measured in a polished section of Murchison (MURCH-2) prepared using Al_2O_3 as the abrasive.

As shown in Table 1, nine of the twelve grains measured showed significant ¹³C enrichments. The enrichments are lower limits on the true values; the grains are at the limit of that which can be measured, and the isotope signals are diluted by unknown amounts of C and Si sputtered from the surrounding material. The contribution will be highest for the more abundant Si, and for this reason we have not tabulated the Si isotope data. With one exception, the Si isotope ratios are close to normal, as would be expected from the dilution of the signal. Murchison grain #10 had $\delta^{29}\text{Si} = -108 \pm 4\text{‰}$ and $\delta^{30}\text{Si} = 71 \pm 10\text{‰}$. In comparison with results from acid residues⁵ the Si isotopic composition of this grain is unique. The C/Si ratio for this analysis was close to unity (0.9), as would be expected of a pure SiC grain.

Figure 1 shows an example of an indigenous SiC grain in CB-4. Although there was an extended bright Si signal in the X-ray maps, neither secondary nor back-scattered electron images showed an identifiable grain in this region (Fig. 1*a*). The underlying SiC grain became clearly apparent when the surface was sputtered away (Fig. 1*b*). In every other case, the X-ray maps could be associated with an identifiable SiC grain before the ion-probe analysis. Figure 2 shows an example of an isotopically anomalous SiC grain in MURCH-2.

The results on CB-4 and MURCH-2 are in marked contrast with those obtained earlier by us on another section of Cold Bokkeveld prepared at the British Museum (CB-1). In that

TABLE 1 Ion-probe analyses of SiC grains

Section CB-4		
Grain	Size (μm)	$\delta^{13}\text{C}$ (‰)
359	1.5 $\times$ 0.75	+518 $\pm$ 37
671	1.0 $\times$ 2.9	+440 $\pm$ 7
887	1.6 $\times$ 1.7	+3 $\pm$ 19
889	1.6 $\times$ 2.7	-25 $\pm$ 2.3
997	1.7 $\times$ 1.4	+568 $\pm$ 53
1,311	1.4 $\times$ 1.4	+779 $\pm$ 29
1,390	1.6 $\times$ 1.6	+16 $\pm$ 8
1,446	1.2 $\times$ 1.9	+2,800 $\pm$ 160
Section MURCH-2		
Grain	Size (μm)	$\delta^{13}\text{C}$ (‰)
8	1 $\times$ 1	199 $\pm$ 26
10	2 $\times$ 2	742 $\pm$ 14
409	2.5 $\times$ 2.5	468 $\pm$ 4
609	1 $\times$ 1	314 $\pm$ 25

The isotopic compositions are minimum values owing to a contribution from the matrix surrounding the grains. Although contamination by terrestrial SiC ($\delta^{13}\text{C} = -25\text{‰}$) is a problem, the C isotopic compositions of all but three of the grains clearly demonstrate they are indigenous. $\delta^{13}\text{C}(\text{‰}) = [(^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}}] - 1 \times 1,000$, where standard is PDB.

section the concentration of SiC was much higher, all of the grains were associated with cracks and holes, and the isotope ratios were normal. It turned out that CB-1 had been exposed to SiC abrasives in one stage of its preparation. Contamination is thus a problem and the only certain method of identifying indigenous grains is to show they are isotopically anomalous.

Three of the grains in Table 1 have normal carbon isotopic compositions. In each of these cases the petrological evidence for an indigenous origin was ambiguous, and it is possible that they are terrestrial contaminants. We do not mean to imply that all SiC grains with normal carbon isotopic compositions are necessarily contaminants. Previous measurements in acid separates have shown that there are grains with normal C but anomalous Si. There are also a limited number of grains that are normal in both C and Si but anomalous in N (ref. 5). In related work⁸ we have found that almost all SiC grains in an acid residue of the CV meteorite Leoville have normal C and Si. Numerous SiC grains found in acid residues of unequilibrium ordinary chondrites are also isotopically normal⁶⁻⁸ (N was not measured in these cases). As care was taken to avoid contamination, we believe that there is a population of isotopically normal SiC grains in primitive meteorites. The existence of indigenous SiC grains with normal isotopic compositions would raise the question of whether SiC formed in certain regions of the solar nebula; however, this would not obviate the arguments for an interstellar origin of the isotopically anomalous components.

We estimated the concentration of SiC in CB-4 from the areal fraction of SiC on the section. The detection efficiency for small grains was determined empirically by making repeated runs on the same grains and determining the fraction of the maps in which we found grains. The detection efficiency is unity for grains larger than 1.4 μm in diameter but drops rapidly to ~ 0.1 for grains 0.6 μm in diameter⁹.

Counting all SiC grains found, the CB-4 data give a concentration of ~ 3 p.p.m. for grains $> 0.6 \mu\text{m}$ in diameter. For comparison, dissolution experiments in Murchison give 3.5 p.p.m. for grains $> 0.2 \mu\text{m}$ in size⁵. Given the uncertainties inherent in comparing different meteorites, coupled with the possibilities of contamination and the undetermined amount of grain plucking during polishing of CB-4, we consider the agreement to be reasonable. More accurate measurements of mass-size distributions of both *in situ* and residue grains are, however, desirable.

We carefully examined the area around each of the *in situ* SiC grains for associations with other minerals. As far as we

could determine, each of the nine isotopically anomalous grains occurred as isolated matrix particles. This result does not prove that such associations do not exist. For example, loose clusters of 1- μm SiC intermixed with other material would not be efficiently detected by X-ray mapping. This is because the maps would have to be made at high magnification to locate the small SiC grains and this would restrict the total area scanned. The fact that the concentration we determined is approximately the same as that found in the residues shows that most of the SiC does not occur in such hypothetical clusters, but we cannot rule out the possibility that a significant fraction of the SiC could be present in this form. Nor can we rule out the (unlikely) possibility that the oxygen ashing treatment may have removed carbon-rich associated grains. We note that none of the SiC grains occurred as inclusions in larger grains—a possibility that would have explained their survival in the solar nebula despite being thermodynamically unstable under nebular conditions. We note that CM meteorites show substantial hydrothermal alteration^{15,16}. Unequilibrated ordinary chondrites, which are less altered and which are also known to contain isotopically anomalous SiC⁶⁻⁸, may be a more promising place to look for interstellar grain associations.

It is natural to enquire whether SiC grains are altered by the harsh chemical and physical treatments that are used to produce acid residues. The *in situ* grain shown in Fig. 2 is extensively cracked and such a grain might break up during processing. Similar cracks are seen in several of the other *in situ* grains. The cracks could be a polishing artefact, but neither our data nor published residue data are sufficiently detailed to establish whether there is a substantial difference in the grain size distribution functions of the SiC. The fact that different size fractions of SiC grains from residues have distinctly different isotope properties^{3,5} indicates that most of the residue grains are probably not derived from larger ones.

The mapping protocol would have located grains of certain other silicon-rich minerals, specifically silicon nitrides, had they been present in the same size range and at a concentration $> 10\%$ of the SiC. Indeed such grains have been found recently in acid residues of the reduced enstatite chondrites Indarch¹⁷ and Qingzhen (C. A. *et al.*, unpublished data). We did not find such grains in the CM meteorites studied here.

We undertook this work in the hope that SiC would prove a useful tracer for pre-solar materials that might have been destroyed in the etching treatments. Although this has not so

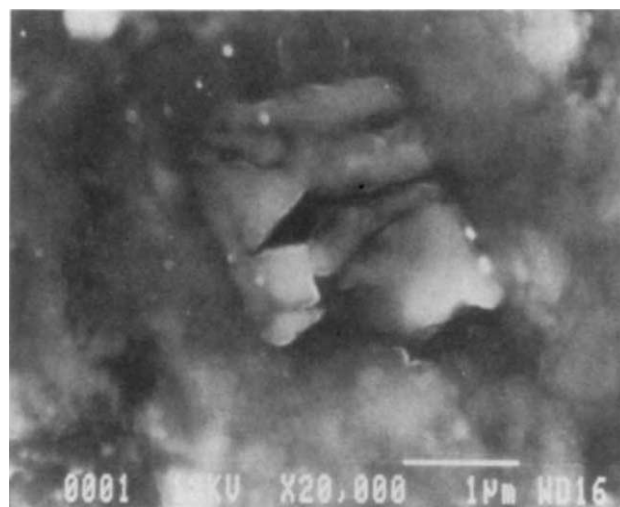


FIG. 2 A fissured grain of SiC (MURCH-2-10), *in situ*, in Murchison. Although the cracks and fissures may be polishing artefacts, their regular appearance suggests that they represent inherent structural features.

far proved to be the case, we intend to continue to search for associations of SiC with other minerals in a wider variety of meteorites. It is also important to improve the measurements of the mass distribution function of both *in situ* and acid-residue grains to permit a better quantitative comparison of the two. It may be possible to extend mapping techniques to locate other types of interstellar grains, for example, the morphologically distinct graphite grains recently discovered in acid residues¹⁸. Of course, the ultimate mapping technique would use isotope images to permit detection of *in situ* grains from both carbon-rich and oxygen-rich stars. □

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Structure of a fossil ridge–transform intersection in the Troodos ophiolite

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RECENT palaeomagnetic studies of sheeted dykes adjacent to the east–west trending Southern Troodos transform fault of Cyprus have demonstrated significant clockwise block rotations, which are most easily interpreted as being due to drag induced by dextral strike-slip faulting along the transform^{1–6}. We show here that such rotations have not occurred along the entire length of the transform; instead, to the west of the areas previously studied the sheeted dykes have been subjected to simple tilting only, indicating normal faulting but no strike slip. The changeover between these two domains of differing structural styles, near the village of Mandria, is approximately in line with the centre of the Solea graben, a north–south trending structure within the Troodos massif which has previously been interpreted as a fossil ridge axis⁷. We suggest that the Mandria area represents a fossilized ridge–transform intersection, and present the results of a detailed field investigation of the area, together with preliminary palaeomagnetic data, to determine the relationship between tectonism and magmatism at such plate boundary zones. We use our findings to test a recent theoretical model⁸ of deformation processes at ridge–transform intersections, and show that distributed deformation due to block rotation is largely confined to the ridge–transform corner itself.

The Southern Troodos transform fault (formerly called the 'Arakapas fault zone'⁹) is an approximately 5-km-wide oceanic strike-slip fault system trending E–W along the southern margin of the ophiolite^{9–11}. To the south a fragment of crust generated at an 'Anti-Troodos' ridge axis has been recognized⁹. Within the transform, ophiolite-derived debris flows and turbidites intercalated with lava flows attest to the existence of a deep and irregular E–W-elongate bathymetric depression coincident with the fault zone^{9,10}.

The Solea graben is a N–S-trending fault-controlled structure, 20–40 km wide, in the Troodos massif that is parallel to the average dyke strike in the sheeted complex and by implication parallel to the spreading ridge (Fig. 1a)^{7,12}. It is defined by the tilt of rotated blocks such that they dip towards the graben axis, the dykes having been rotated by faults along near-horizontal axes parallel to the axis of the graben¹³. The association of sulphide mineralization with these faults¹⁴, and the near-horizontal disposition of overlying Upper Cretaceous to Middle Miocene Lefkara Group pelagic chalks, demonstrates that the graben formed in an oceanic environment.

Within the Troodos massif, to the south and east of Mount Olympus, the N–S attitude of the sheeted dyke complex is rotated progressively to E–W, over a distance perpendicular to the transform of 5–15 km, as the transform is approached (Fig. 1a)¹⁰. This swing of dykes has been interpreted either as an original feature of the spreading fabric, reflecting dyke intrusion in a sigmoidal stress field at a sinistrally slipping transform system^{7,11,12,15}, or as a result of the physical block rotation of originally N–S-trending dykes by fault drag at a dextrally slipping transform¹⁰. Recent palaeomagnetic studies of these dykes have shown that their primary magnetization vectors change as the dyke strikes change, demonstrating beyond reasonable doubt that the dyke swing is a secondary feature imposed on originally N–S dykes^{2–4,6} and confirming the latter model.

A few kilometres to the west of Mandria, the swing in dyke strike disappears. Further to the west the dykes trend uniformly N–S throughout (Fig. 1a, b). Figure 1b illustrates the trends of dykes both of the sheeted complex and those intruding and feeding the related lower or 'axis sequence' pillow lavas in this region. To the east, dykes swing from N–S to E–W as the transform is approached, as described above for the 50 km or so to the east. Our palaeomagnetic results (Table 1, Figs 1b and 2) confirm the earlier studies^{1–6} from the dykes east of Mount Olympus and show that the NE-trending dykes have been rotated clockwise by up to 70° about steeply inclined axes, from an original N–S or NNW–SSE intrusion direction.

In marked contrast, palaeomagnetic results from the N–S-trending dykes in the western part of this area (Table 1, Figs 1b and 2) indicate that they have not been rotated significantly from their original intrusion directions, showing small degrees of simple tilting about gently plunging axes, consistent with normal faulting.

A palaeomagnetic site (C12) from a massive boss that intrudes rotated lavas (with NE-trending dykes) in the eastern domain (near site C6; see Fig. 1b) is itself unrotated, demonstrating that rotation of the dykes occurred at an early stage in the formation of the Troodos oceanic crust.

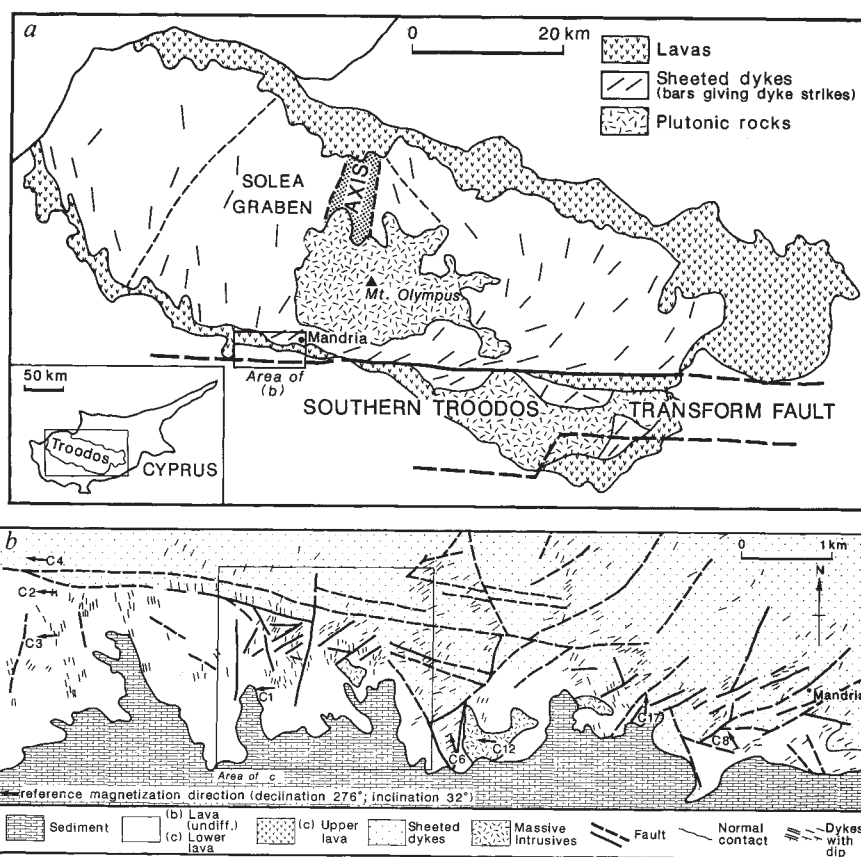
The changeover from unrotated (N–S) to rotated (070°-striking) dykes occurs across a zone about 2–5 km wide (in an E–W direction) centred 4–5 km west of Mandria (Fig. 1b, c). This zone marks the boundary between an eastern domain of dykes rotated by drag due to dextral strike-slip adjacent to the transform fault and a western domain of N–S dykes affected by normal faulting only. No major late-stage displacive fault system exists between the two domains to account for the difference in structural style (see Fig. 1b and below), hence we conclude that the relationship between them is a function of the original oceanic spreading fabric. The partition of structural styles is as predicted for a classic oceanic ridge–transform intersection¹⁶ (Fig. 3a). We therefore deduce that the Mandria area represents

TABLE 1 Palaeomagnetic data from sites in the Mandria region

Site	Dec	Cleaned magnetization vector				Dyke orientation			Unit
		Inc	<i>k</i>	α_{95}	<i>N</i>	Strike	Dip	Sense	
C1	273	50	6.0	19	9	—	—	—	upper pillow lavas
C2	284	26	8.9	21	5	351	75	W	lower lavas and dykes
C3	262	33	40.3	9	6	014	60	W	lower lavas and dykes
C4	298	25	8.2	20	6	016	62	W	lower lavas and dykes
C6	002	59	17.4	15	5	071	56	S	sheeted dykes with lava screens
C8	337	13	57.1	7	7	078	75	N	sheeted dykes with lava screens
C12	283	33	298.0	4	5	—	—	—	upper lava feeder centre
C17	009	32	51.0	8	6	057	60	N	sheeted dykes

Locations of sites are indicated on Fig. 1*b*. Samples were progressively demagnetized using either thermal or alternating-field techniques^{2,4,13}. No tilt corrections have been applied. *k*, Fisher precision parameter; α_{95} , angle of cone of confidence at 95% probability; *N*, number of samples per site. Results should be compared to a regional reference direction of declination 276°, inclination 32°, following the ~90° anticlockwise rotation of the entire Troodos massif^{17,18}.

FIG. 1 *a*, Troodos ophiolite, Cyprus, showing major lithologies and principal elements of the spreading structure: the Solea graben (fossil spreading axis) and Southern Troodos transform fault. The Mandria area lies at the intersection of the graben and the transform fault. *b*, Geological map of the Mandria area (location shown in *a*), with palaeomagnetic sites shown (declinations indicated by arrows). The declinations can be compared with the regional reference direction of (276°, 32°), which results from the 90° anticlockwise rotation of the entire ophiolite on emplacement^{17,18}. *c*, Detail of *b* in the critical zone, across which dykes change strike from N-S (no block rotation) to ENE-WSW (significant block rotation). The transition from N-S to ENE-WSW strike occurs both up section and from east to west within the lower pillow lavas. Simplified from 1:5,000 field mapping; for clarity, not all dykes and cross-cutting relationships are shown.



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Anticrack-associated faulting at very high pressure in natural olivine

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SHALLOW earthquakes are produced by brittle shear fracture of rock and/or frictional sliding on pre-existing fault surfaces¹. At very high pressures, however, brittle fracture and frictional sliding are impossible because frictional resistance to movement on any potential fault surface is so great that ductile stress-relief processes accommodate strain at lower shear stresses than those necessary to activate faulting. Nevertheless, more than 20% of earthquakes with magnitudes greater than five occur at depths greater than 300 km, where the pressure exceeds 10 GPa (ref. 2). Possible explanations for this paradox have been offered by several recent experimental studies of shearing instabilities associated with phase transformations at high pressure^{3–8}. One of these mechanisms is associated with anticrack development during the olivine → spinel ($\alpha \rightarrow \gamma$) phase transformation in Mg_2GeO_4 at pressures of 1–2 GPa (refs 4–6); it is particularly attractive because it operates between mineral structures known to occur in the mantle. We show here that this mechanism also can operate in natural silicate olivine, $(\text{Mg, Fe})_2\text{SiO}_4$, during onset of the $\alpha \rightarrow \beta$ transformation at the much higher pressures at which deep earthquakes occur. These results lend strong support to the hypothesis that the anticrack mechanism is responsible for such earthquakes⁴.

In the experiments with the germanate analogue, faulting occurred only in a very narrow temperature interval corresponding to the kinetically controlled onset of transformation. A unique set of microstructures characterized faulted specimens. In particular, throughout the specimens, microscopic lenses of extremely fine-grained ($\ll 1 \mu\text{m}$) spinel (mode I anticracks) developed normal to the maximum compressive stress during

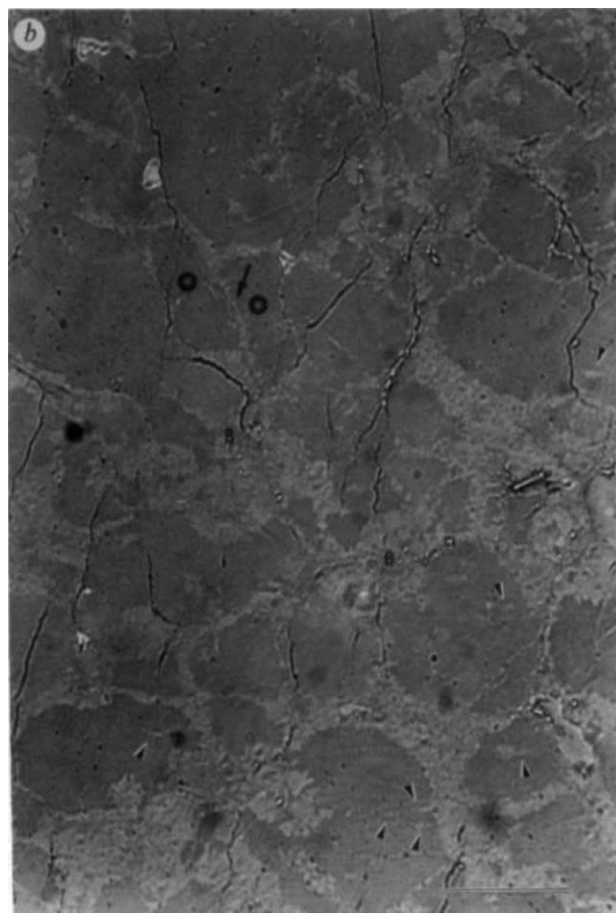
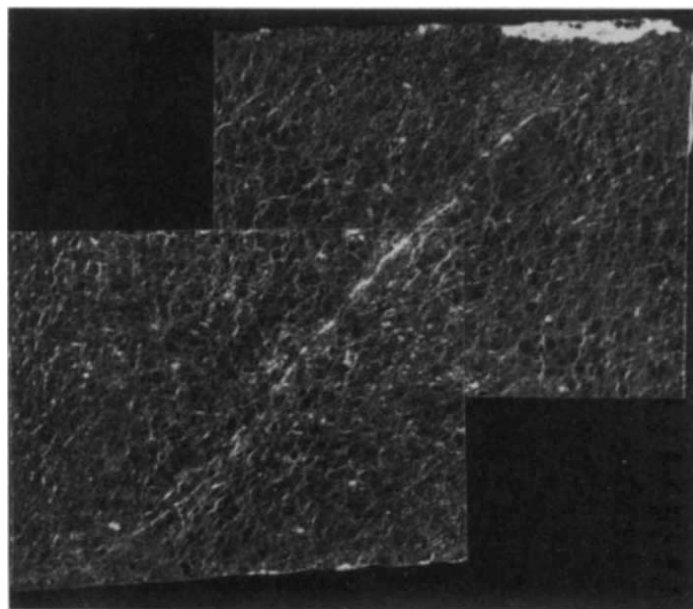


FIG. 2 Photomicrographs in *a*, transmitted light with crossed polarizers and *b*, vertically incident reflected light, showing microstructures associated with the fault in specimen 36. *a*, Regions of extensive transformation to the high-density β -phase show very low birefringence and are labelled β . The grain-boundary regions of all olivine crystals and intracrystalline lens-shaped regions (arrowheads) show reduced birefringence due to growth of the β -phase. The lenses have a strong preferred orientation in the E-W direction. The fault zone runs from upper left to lower right, producing a right-lateral offset in an olivine crystal in the upper third of the field of view (stars). *b*, In reflection, the β -phase is brighter than olivine; its distribution on all olivine grain boundaries, in the fault zone and in the lenses (arrowheads) is readily apparent. The offset (arrow) in the olivine crystal (stars) is particularly obvious as are the very thin regions of β -phase along the fault in that crystal (arrow). Scale bars, 50 μm .

FIG. 1 Low-magnification photomicrograph in obliquely incident light showing a fault traversing specimen 36. The specimen was shortened in approximately the N-S direction. Bright areas on the edges of the specimen are remnants of the rhenium heater. Horizontal dimension of specimen is 3 mm.



loading and faults were found to contain similarly fine-grained spinel. These experiments demonstrated that fault propagation by this mechanism is a microscopically ductile process⁶ and led to the proposal that analogous faulting in the relatively cold interiors of subducting lithospheric slabs is responsible for deep-focus earthquakes^{4,5}.

Despite the advantages of the anticrack faulting model, some important questions could not be answered by the germanate study: (1) Can this mechanism really operate at very high pressure? The extremely fine grain size of the Mg_2GeO_4 spinel in fault zones suggested that superplastic flow⁹ was the mechanism accommodating faulting but faults formed at $\sim 30^\circ$ to the direction of maximum compression and through-going faults with significant slip displayed brittle as well as ductile microstructures. (2) Is the germanate analogue reliable? Mg_2GeO_4 undergoes isochemical transformation directly from the olivine (α) phase to the spinel (γ) phase. In the natural silicate system, however, the spinel (β) phase intervenes between α and γ , and two-phase stability fields occur with preferential partitioning of iron into the denser phases^{10,11}.

To answer these questions we have begun a series of deformation experiments on a synthetic harzburgite consisting of 96% olivine, 3% orthopyroxene and 1% chromite^{12,13} in the new multianvil apparatus at Lamont-Doherty¹⁴. Cylindrical specimens, 3 mm in diameter by ~ 6 mm long, were placed between Al_2O_3 end pieces ~ 2 mm long and inserted inside rhenium tube heaters of an MA6/MA8 assembly. We used octahedral facets (truncated edge length of 6 mm) on cubic anvils to compress an integral pressure medium/gasket assembly cast from uniform Aremco 584 potting compound. Although the multianvil apparatus is designed to produce hydrostatic conditions, non-hydrostatic stress was achieved by using the Al_2O_3 end pieces as pistons and choosing the length of the specimens such that they would be under quasi-axial compression during pressurization.

Using the microstructures seen in the germanate specimens as a guide, we systematically altered specimen lengths and/or the experimental parameters from one run to the next to search for conditions that would yield anticracks. The purpose was twofold—to see if conditions of anticrack development could be found and to see if faulting would occur under such conditions. In all experiments, pressurization to 9 GPa was performed at room temperature, the temperature was raised to the desired level and pressurization was resumed for ~ 10 minutes. When 15 GPa was reached, pumping was terminated and the specimen

was quenched by cutting power to the furnace. The sample was then slowly depressurized over ~ 12 hours. Of the seven experiments attempted, three failed for technical reasons, three provided useful information for choice of subsequent experimental conditions, and the seventh achieved the goal. Specimen 29 was too long and fractured during early pressurization. The other three successful experiments, 31, 35 and 36, conducted at nominal central temperatures of 1,650, 1,650 and 1,550 K, respectively, showed contrasting microstructures. Specimen 31, for which we used crushable Al_2O_3 pistons, lacked the microstructures typical of transformation under non-hydrostatic stress (see below); apparently it was under quasi-hydrostatic pressure. Subsequent experiments were conducted with sintered Al_2O_3 pistons and specimens 35 and 36 both displayed microstructures indicative of non-hydrostatic stress. The nominal axial strain rate was $\sim 2 \times 10^{-4} \text{ s}^{-1}$, roughly comparable to the germanate experiments⁴⁻⁶.

The central regions of specimens 31 and 35 show equant and strain-free olivine grains that are significantly smaller than those of the starting material, indicating extensive recrystallization. Both specimens also contain a weakly birefringent phase of high refractive index on grain boundaries throughout their volumes, including the central regions of recrystallized olivine. Specimen 31 shows no evidence of non-hydrostatic stress. The high-refractive-index phase nucleated equally on grain boundaries of all orientations and shows no preferred growth direction. Specimen 35 shows somewhat less recrystallization of olivine in the centre, suggesting a slightly lower maximum temperature than specimen 31; the high-refractive-index phase is more abundant than in specimen 31, especially near the (colder) ends of the specimen where it constitutes $\sim 80\%$ of the rock. In these regions, nucleation of this phase occurred preferentially on grain boundaries normal to maximum compression σ_1 , and crystals grew elongated parallel to σ_1 . From its optical properties we identify this phase as β -olivine.

Specimen 36 shows recrystallization of olivine only in the central (hottest) third of the specimen and the 'rind' of β -olivine on grain boundaries is everywhere thinner than in specimen 35. Specimen 36 also contains a fault that cuts completely through the specimen (Fig. 1), traversing both the regions where olivine did not recrystallize and the central zone where it did. In general, the fault cannot be located precisely near the specimen edges because of its small displacement and extensive transformation adjacent to the heater. Figure 2 shows a segment of the fault trace that illustrates the critical microstructures. Between crossed

polarizers (Fig. 2a), the low birefringence of the β -phase is illustrated in the grey areas labelled β and by the reduction of birefringence around the boundaries of (and locally within) olivine grains. The fault zone cuts diagonally across the field of view at an angle of $\sim 30^\circ$ to the N-S direction of the micrograph. It cuts and offsets by $20\ \mu\text{m}$ in a right-lateral sense the cream-coloured olivine crystal marked with stars. Elsewhere in this field of view, the fault mostly follows grain boundaries, but we have identified in two thin sections about 10 other crystals that have been similarly offset by $10\text{--}30\ \mu\text{m}$. Significantly, two of the offset crystals are recrystallized olivine in the central portion of the sample. Several of the olivine crystals near the fault in Fig. 2a display subparallel lens-shaped regions of reduced birefringence that trend E-W in the micrograph (arrowheads). They are particularly well displayed in two of the three blue olivine crystals in the lower half of the micrograph.

In vertically incident reflected light (Fig. 2b), the β -phase is brighter than olivine. It is present on all olivine grain boundaries and it fills the fault zone. The fault trace is free from any cracks throughout the segment illustrated except for one small region just above the offset olivine crystal. Note in particular that this and other cracks visible in Fig. 2 cut directly across high-temperature features. Where the fault traverses the offset olivine crystal, it consists of thin ($\sim 1\ \mu\text{m}$) anastomosing zones with small regions of olivine in between (arrow). Lenses that are exposed at the surface (arrowheads) are also bright and therefore are composed of the β -phase.

It is critically important to determine when faulting occurred in specimen 36. Was it the result of brittle fracture during the early stages of pressurization at room temperature or did it occur in association with the subsequent phase transformation at high temperature and pressure? If the fault formed at low pressure by brittle processes, large numbers of mode I microcracks parallel to σ_1 would have developed also and cracking associated with the fault zone would have occurred. These cracks, in turn, would have served as nucleation sites for both olivine and β -phase after the temperature was raised. In particular, the fault would be completely healed and invisible in the region of recrystallized olivine because the recrystallization can only have occurred after the initial pressurization, during heating. Elsewhere, the 'rind' of β -phase on cracks and along the fault trace would be as thick and irregular as on grain boundaries. Alternatively, if faulting occurred near the end of the experiment, at high pressure, the fault should be well defined through the zone of recrystallized olivine and, if the mechanism of faulting was the anticrack instability⁴⁻⁶, the characteristic microstructures associated with that instability also should be present.

Our observations provide conclusive evidence that faulting occurred late in experiment 36. Figure 2 shows that all cracks are late structures that cross-cut high-temperature features indiscriminantly. Similar cracks occur throughout specimens 31 and 35; they must have formed during decompression. Only rarely do we find olivine crystals cut by zones of β -phase that might be evidence of early microcracks that have been overgrown at high temperature. In contrast to what would be expected for an early fault, the fault is a remarkably sharp feature that crosses the entire specimen, including the recrystallized olivine zone, and lacks evidence of overgrowth. Lastly (and definitively), the fault cuts and offsets recrystallized olivine grains that grew at a pressure $>9\ \text{GPa}$ and displays the denser phase in the very narrow fault zone.

The microstructures of all three specimens correspond very closely to those produced under comparable conditions in Mg_2GeO_4 . In the germanate, transformation at high temperature is accomplished by incoherent nucleation and growth^{15,16}. Under quasi-hydrostatic pressure, crystals of the denser phase are equant and are evenly distributed on grain boundaries as they are in specimen 31, whereas under non-hydrostatic stress, nucleation occurs preferentially on olivine grain boundaries normal to σ_1 and growth is faster parallel to σ_1 (refs 17, 18),

as in specimen 35. At somewhat lower temperatures in the germanates, where transformation to the high-pressure phase is slow on the timescale of the experiment, the anticrack lenses of the denser phase exhibit a strong preferred orientation and are found in association with microscopically ductile shear failure in which the resulting fault zone is filled with the denser phase⁴⁻⁶, as in specimen 36. In the germanate, anticracks visible optically are invariably accompanied by very much larger numbers of submicroscopic ones that develop on all grain boundaries. We conclude that faulting in the silicate occurred at high temperature and pressure by the same anticrack-associated mechanism as in the germanate, possibly in association with (and the cause of) a brief power disturbance at $14\ \text{GPa}$ that occurred in experiment 36.

Thus we conclude that we can use the results of the germanate experiments for interpretation of these silicate specimens. In particular, the lenses developed in the olivine crystals of specimen 36 are morphologically indistinguishable from the lenses found in germanate specimens that faulted (compare Fig. 9a of ref. 5 and the cover of ref. 4). In the germanate, the lenses are mode I anticracks that form normal to σ_1 and are filled with extremely fine-grained γ -phase. We conclude, therefore, that the lenses in specimen 36 are β -phase-filled mode I anticracks aligned perpendicular to σ_1 . In contrast to the germanate experiments, in which the orientation of the anticracks is constant throughout the specimens (reflecting the homogeneous state of stress in those experiments), the anticracks in specimen 36 are parallel to each other locally but vary systematically over the specimen, reflecting the inhomogeneity of the stress field in these more complicated experiments. The anticracks adjacent to the fault zone in Fig. 2 establish the local orientation of σ_1 to be N-S in the micrograph and indicate that, as in the case of the germanate⁴⁻⁶, faulting associated with anticrack development occurs at $\sim 30^\circ$ to σ_1 . Although we have not yet been able to resolve the grain size in the anticracks or fault of specimen 36, the fact that the thickness of the transformed zones in the fault are as thin as $\sim 1\ \mu\text{m}$ provides an upper bound on the grain size. From the germanate studies, we estimate the grain size to be at least an order of magnitude finer than that.

Our observations provide first definitive evidence of fluid-absent faulting at very high pressure. The $\alpha \rightarrow \beta$ and $\alpha \rightarrow \gamma$ olivine phase transformations responsible for the instability occur in mantle peridotite during subduction of oceanic lithosphere at convergent plate margins. It is probable that these transformations are kinetically inhibited in the cold interior of downgoing slabs and that when the rocks warm to the threshold of nucleation of the denser phases under stress, faulting is triggered and deep-focus earthquakes are generated. We are continuing our studies on both silicate olivine and its analogues to clarify the mechanism by which anticracks participate in the faulting process and to establish the seismic signal emitted during faulting. □

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Nonadaptive clutch sizes in tits

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ALTHOUGH Lack^{1,2} proposed that birds should lay clutches of a size that produces the most recruited offspring, in reality the most frequent clutch size tends to be smaller than the most productive one³. Of the six different but not mutually exclusive hypotheses explaining why Lack's hypothesis^{1,2} is not correct, four are based on adaptive explanations³⁻⁸ whereas two are nonadaptive⁹⁻¹¹. One of the latter argues that because of gene flow between habitats of different quality and hence different optimal clutch sizes, clutches may be larger than optimum in poor habitats and smaller than optimum in good habitats^{9,10}. This is the only hypothesis for which no evidence has been obtained up to now. We now present evidence that great and blue tits (*Parus major* and *P. caeruleus*) do not adjust their clutch size in an adaptive way in habitats of different quality. The results support the 'nonadaptive' hypothesis that because of gene flow between habitats of different quality as a result of birds moving from one habitat to another, birds can be unable to produce a clutch size adapted to the habitat in which they breed^{9,10}.

We tested the gene flow hypothesis by comparing the most frequent and the most productive clutch size for great and blue tits in a series of study sites differing in quality. In all populations a large proportion of the breeding birds had immigrated from outside, where habitat quality may differ. Habitat quality was expressed as the proportion of eggs producing fledglings (fledglings per egg, f/e) in successful first broods averaged over many years. For most study areas data on nestling weight at day 15 were also available. This is similar to fledgling weight, reflects the amount of food parents can provide, and is a good predictor of post-fledging survival^{12,13}. Nest mortality in tits breeding in nest-boxes is mainly caused by starvation of young in the nest. Nestling weights were lower in plots in which f/e was lower, supporting the validity of the criterion used (using the one-tailed Spearman rank correlation coefficient: great tit $r_s = 0.988$, $n = 5$, $P < 0.05$; blue tit $r_s = 1.0$, $n = 4$, $P = 0.05$ (Table 1)). For the two species a large and parallel between-habitat variation exists (see also ref. 14) in each year¹⁵, for both fledglings per egg and fledgling weight, further supporting the validity of the measure of habitat quality used. No differences in weight or size existed between adults breeding in different study plots in the same year (our unpublished results), and experimental studies could not establish a relationship between adult bird quality and territory quality¹⁶.

The most frequent clutch size in great tits was 9 or 10 in each of the study areas, but the most productive one varied between ≥ 12 in plot B and 8 in plot Z. For blue tits the most frequent clutch size was 11 or 12 in each of the study areas, but the most productive one varied between ≥ 14 in plots TL and B, and 9 in plot H (Fig. 1a, Table 1).

We performed pairwise between-plot comparisons on clutch size frequency distributions and on the frequency distributions of recoveries per clutch size, using two-tailed two-sample Kolmogorov-Smirnov tests. If clutch size distributions do not differ between plots but recovery distributions do, then clutch size is not tracking local optima. Where clutch size distributions do not differ, the recovery distributions for the different clutch sizes do differ in the direction predicted, in both species (Table 2). There is a positive relationship between habitat quality and the difference between the most productive and the most frequent clutch size: in good habitats tits lay a clutch that is 'too small', that is, smaller than optimum, whereas in poor habitats they lay a clutch that is 'too large', that is, larger than optimum (Fig. 1b). The data thus support the gene flow hypothesis.

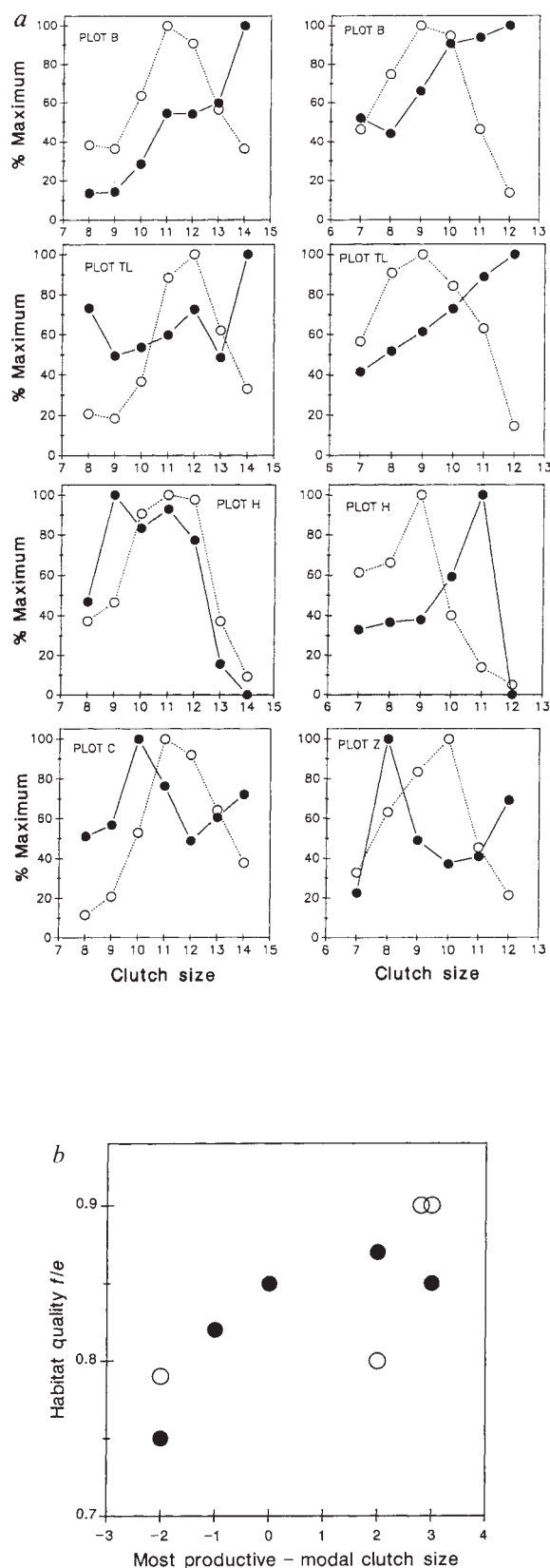


FIG. 1 a, Frequency distributions of clutch size (○) and of recoveries for each clutch size category (●) in different plots, expressed as a percentage of the largest value. Left: blue tit, right: great tit. b, Most productive clutch size as a deviation from the modal clutch size in great tit (○) and blue tit (●) in relation to habitat quality. The Spearman rank correlation coefficient for all nine points is $r_s = 0.84$, one-tailed $P < 0.01$. In better quality habitats the most productive clutch is larger than the most frequent one, whereas in poorer quality habitats the opposite is true.

FIG. 2 Nestling weight at day 15 in relation to clutch size. All years for which weights are available are combined (11 years for plots B, T and C; 4 years for plot Z). Plot T is a 12.5-ha part of plot TL. The largest and smallest clutch sizes include all more extreme clutches. In both tit species young in poorer habitats are both lighter and show a more pronounced decrease in mean weight as a function of clutch size. Since post-fledging survival increases with weight, these relationships provide the mechanism to explain why the optimal clutch size differs between areas of different quality. The slope of the regression equations expressing nestling weight as a function of clutch size are as follows (P , significance of rejecting null hypothesis $b=0$).

Great tit

Plot B ($n=387$), $b=-0.086 \pm \text{s.e. } 0.038$, $P=0.025$.

Plot T ($n=182$), $b=-0.12 \pm \text{s.e. } 0.047$, $P=0.01$.

Plot C ($n=96$), $b=-0.43 \pm \text{s.e. } 0.083$, $P<0.001$.

Plot Z ($n=85$), $b=-0.66 \pm \text{s.e. } 0.16$, $P<0.001$.

The average of all young in a nest is used as one value. n is thus the number of nests.

Analyses of covariance show that: (1) the slopes differ significantly between the 4 areas: $F=12.72$, $\text{d.f.}=3,742$, $P<0.0001$. (2) Neither the slopes nor the intercepts of the two high quality plots B and T differ. Slopes: $F=0.30$, $\text{d.f.}=1,565$, $P=0.58$; intercepts: $F=2.33$, $\text{d.f.}=1,562$, $P=0.13$. (3) The slopes of the two poor quality plots C and Z do not differ, but the intercepts do. Slopes: $F=1.73$, $\text{d.f.}=1,177$, $P=0.19$; intercepts: $F=27.74$, $\text{d.f.}=1,174$, $P<0.0001$.

Blue tit

Plot B ($n=304$), $b=-0.030 \pm \text{s.e. } 0.019$, $P=0.11$.

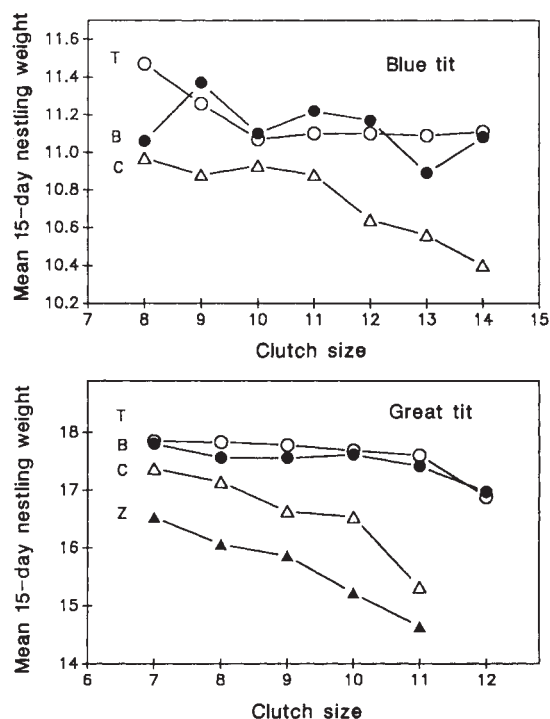
Plot T ($n=258$), $b=-0.039 \pm \text{s.e. } 0.023$, $P=0.09$.

Plot C ($n=396$), $b=-0.11 \pm \text{s.e. } 0.025$, $P<0.001$.

An analysis of covariance shows that the slopes differ between the three areas: $F=3.81$, $\text{d.f.}=2,952$, $P=0.02$.

The reason why larger clutches, although producing more fledglings, sometimes produce fewer recruits than smaller clutches is that weight at fledging tends to decrease with brood size¹². Fledgling survival increases with 15-day nestling weight (refs 12, 13 and our unpublished results), but the latter decreases with brood size (Fig. 2). The most productive clutch size is that in which the number of fledglings times the recruitment probability per young is largest. In both species nestling weights in the poorer study plots are both lower and decrease more rapidly with clutch size than in better plots (Fig. 2). These relationships explain why the optimal clutch size differs between plots.

The mechanism postulated to explain the maladaptive clutch



size is gene flow between populations with different optima^{9,10}. In all populations, both in this and other tit studies, a large majority of breeding birds were not born locally, and some birds born in one plot were found breeding in another one (ref. 17 and our unpublished results). The data are, however, insufficient, to compare recruitment per clutch size for birds of different origin.

Although these results do not exclude various adaptive explanations, such as a survival cost for the adult in raising a large brood^{3,4}, or that the geometric rather than the arithmetic mean should represent the adaptive clutch size over a number of years⁵, or that there is individual optimization^{6,7}, they emphasize

TABLE 1 Modal and most productive clutch sizes for great and blue tits in a series of study plots, ranked by quality during the breeding season

Study plot	Period	No. years	No. nests	No. recoveries	f/e	Fledgling weight (g)	Most frequent clutch size	Most productive clutch size
Great tit								
TL	1979-88	10	311	292	0.90	17.58	9	≥ 12
B	1979-88	10	357	330	0.90	17.64	9	≥ 12
C	1979-88	10	106	13	0.86	16.76	9	—
H	1964-76*	11	229	213	0.80	16.02	9	11
Z	1963-74	12	274	98	0.79	15.65	10	8
Blue tit								
TL	1979-88	10	312	153	0.87	11.16	12	≥ 14
B	1979-88	10	272	163	0.85	11.15	11	≥ 14
G	1972-77	6	112	36	0.85	—	12	12
C	1979-88	10	369	168	0.82	10.73	11	10
H	1963-74	12	162	54	0.75	10.6†	11	9

*Quality is defined as the proportion of eggs giving rise to fledglings (f/e), in first broods producing at least one fledgling, averaged over the number of years indicated. Number of years: that for which recoveries are available and used to calculate the most frequent and the most productive clutch sizes. The number of years for which data on nestling weight are used is indicated in the legend to Fig. 2. No. nests: the total number of first broods from which young fledged in the years used. No. recoveries: the number of individuals alive at least 3 months after fledging. f/e : fledglings per egg is the proportion of eggs producing fledglings in successful first broods. It is related to fledgling weight. Fledgling weight: mean of the nest means of 15-day nestling weight of first broods. Sample sizes are indicated in the legend to Fig. 2. Most frequent clutch size: the mode of the frequency distribution of clutch sizes for all years combined. Most productive clutch size: clutch size from which the highest average number of recoveries per nest was made. ' $\geq$ ' means that larger clutches were at least as productive as the maximum indicated, and were therefore included in the category given. In plot C, great tit, the most productive clutch size cannot be determined, because of too few recoveries.

Plots TL, B, G and Z have oak and plot H beech as the dominant trees. Plots TL (20 ha) and B (12.5 ha) are parts of the same large forested area; all other plots are habitat islands. Plots B, TL and C lie north of Antwerp; the others are around Ghent, about 60 km to the southwest. More details on the study plots are given in refs 14 and 18 (TL and B) and ref. 19 (others).

* Plot H, great tit: no data in 1970 and 1971; weights only in 1967 and 1969 (39 nests).

† Plot H, blue tit: around 150 nests in period 1978-89 (J. De Laet, personal communication).

TABLE 2 Results of between-plot comparisons (*D* values) of two-tailed two-sample Kolmogorov–Smirnov tests comparing the distributions of clutch size frequencies and those of the number of recoveries for each clutch size

Blue tit						
No. of recoveries		153	163	36	168	54
No. of clutches		TL	B	G	C	H
312	TL	—	0.04	0.05	0.17*	0.33**
272	B	0.11	—	0.12	0.18*	0.33**
113	G	0.05	0.11	—	0.14	0.14
369	C	0.04	0.10	0.05	—	0.23
162	H	0.21**	0.10	0.07	0.19	—
Great tit						
No. of recoveries		292	330	98	213	
No. of clutches		TL	B	Z	H	
311	TL	—	0.05	0.12	0.15**	
357	B	0.04	—	0.17**	0.19**	
274	Z	0.09	0.07	—	0.12	
229	H	0.19**	0.21**	0.28**	—	

The distribution of clutch size frequencies in blue tits is the same in all plots, with the exception of one comparison (TL versus H) but for distribution of recoveries significant differences exist between the two poorer plots (C and H) and the two best plots (TL and B), but not between plots of the same quality. In the good plots the most productive clutch size is the largest one, and is thus larger than the most frequent clutch size. In the poorer plots the most frequent clutch size is larger than the most productive one. In the intermediate plot G the most productive and the most frequent clutch size are the same (see also Fig. 1a).

In great tits, although the modal clutch size is the same as in the other plots, the distribution of the clutch sizes differs between plot H (beech) and the other plots, because relatively more small clutches are laid. The interpretation of the differences in the distribution of recoveries between plot H and the other plots is therefore difficult. The results from poorer plot Z compared with the other plots, however, confirm the blue tit results, and show that although the distribution of the clutch sizes does not differ with plots TL and B, the distribution of the recoveries differs significantly from that in plot B.

* $P < 0.05$; ** $P < 0.01$.

that studies carried out in habitats strongly influenced by man and with large variation in quality must consider the possibility that life-history parameters may not be adaptive. □

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Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons

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MODERATE non-progressive cognitive impairment is a consistent feature of Duchenne muscular dystrophy (DMD)^{1,2}, although no central nervous system (CNS) abnormality has been identified³. Recent studies have elucidated the molecular defect in DMD, including the absence of the protein dystrophin in affected individuals⁴. Normal brain tissue contains dystrophin messenger RNA^{5,6} and dystrophin is present in low abundance in the brain⁷ and seems to be regulated in this tissue, at least in part, by a promoter that differs from that in muscle^{8,9}. Until now, antibodies and immunocytochemical methods used to demonstrate dystrophin at the plasma membrane of mouse and human muscle have proven inadequate to localize precisely dystrophin in the mammalian CNS. We have now made an antibody (anti 6-10) which is much more sensitive than those previously available to immunolabel dystrophin in the CNS. Using this antibody, we found that in the mouse, dystrophin is particularly abundant in the neurons of the cerebral and cerebellar cortices, and that it is localized at post-synaptic membrane specializations. Dystrophin may have a different role in neurons than in muscle, and an alteration at the synaptic level may be the basis of the cognitive impairment in DMD.

As part of our effort to develop more sensitive methods of dystrophin detection, we immunized rabbits with a fusion protein produced from a large segment of dystrophin rod domain. The resulting antibody (anti 6-10) is extremely sensitive, enabling low levels of dystrophin to be detected. Given early difficulty in demonstrating the presence of dystrophin in the brain, we began by dissecting various regions of normal and dystrophin-deficient *mdx* mouse¹⁰ brain to determine the regional distribution of dystrophin. Immunoblotting of these dissected brain regions (Fig. 1) illustrates the specificity of the antibody for dystrophin in normal versus *mdx* mouse and indicates that the cerebellum contains the most dystrophin, followed by cerebral cortex, then brain stem and spinal cord, when standardized by wet weight of tissue. Repeated trials, simultaneous staining of identically prepared immunoblots with antisera to three forms of neurofilament, and protein staining, all suggest that this pattern cannot be ascribed simply to variations in loading of the electrophoresis lanes, but rather that the cerebellum and cerebral cortex are particularly rich in dystrophin.

Inherently, immunoblotting reflects dystrophin in vascular smooth muscle as well as dystrophin intrinsic to neural or glial elements. Immunofluorescence, facilitated by the greater sensitivity of antibody 6-10, permitted the unambiguous visualization of dystrophin in both smooth muscle and neurons in brain sections (Fig. 2a, d). The absence of immunoreactivity in anatomically identical areas in the dystrophin-deficient *mdx* mouse strongly suggests that this labelling represents dystrophin and not another protein (Fig. 2c). There was dense staining of the plasma membrane surfaces of vascular smooth muscle cells (Fig. 2a). In the nervous system proper, the immunoreactivity was confined to two recognizable classes of neurons—the

Purkinje cells of the cerebellum (Fig. 2a) and cerebral cortical neurons (Fig. 2d). In the Purkinje cells there was intense punctate dystrophin immunoreactivity along the membrane of the soma and dendrites, but no intracytoplasmic staining, and no staining along Purkinje cell axons. There was no immunoreactivity in any of the other elements in the cerebellar cortex: granule cells, synaptic glomeruli, stellate or basket cells, or glia (Fig. 2a).

In the cerebral cortex an attenuated version of the pattern seen in the cerebellum was apparent (Fig. 2d). Dystrophin occurred as punctate aggregates along the soma and dendrites of pyramidal neurons. The aggregates were smaller and sparser than in Purkinje cells. Again there was no cytoplasmic staining and no staining that was clearly associated with axons. Although all of the cells that stained had recognizable dendritic architecture and appeared to be of the pyramidal or fusiform type, the possibility that some stellate neurons also contain dystrophin could not be excluded. It also seems likely that not all cortical pyramidal neurons contain dystrophin because in areas such as layers II and III of parietal cortex, predominantly composed of pyramidal neurons, there were many neurons that were non-reactive (not shown). Nonetheless, in other areas also composed principally of pyramidal neurons, such as the deep layers of frontal cortex, there was staining in nearly all neurons, visualized as punctate peripheral immunoreactivity (not shown). As in the cerebellum, glial cells and the white matter were completely non-reactive. In addition, an initial survey of other grey-matter structures such as the striatum, thalamus, hypothalamus and brain stem reticular formation indicated that there is little if any reactivity in neurons of these regions. Preliminary studies of human autopsy brain tissue in dystrophin immunoblots and immunofluorescence suggest qualitatively similar results.

To confirm and extend our immunofluorescence results, immunogold electron microscopy was employed (Fig. 3). In

both cerebral cortical neurons and Purkinje cells groups of gold particles reflecting dystrophin immunoreactivity were found in discrete sub-plasma membrane deposits. Long expanses of plasma membrane showed no labelling. The foci of immunoreactivity were immediately adjacent to short regions of membrane apposition and thickening of the plasma membrane. These features are consistent with synaptic contacts, although the conditions for frozen-section immunoelectron microscopy are not optimal for demonstrating presynaptic vesicles and post-synaptic membrane specializations. Similar profiles were also found without label in normal neurons and no labelling was seen in *mdx* brain (Fig. 3d).

Thus, dystrophin is present in neurons of the CNS, and occurs in a restricted distribution both in terms of cell type and sub-cellular location. Dystrophin is structurally related to the α and β spectrins¹¹, proteins that form a meshwork beneath the plasma membrane of many cell types¹². This spectrin membrane skeleton is thought to provide structural support to the membrane, regulate the distribution of integral membrane proteins and link membrane elements with other cytoskeletal components such as actin filaments¹². In muscle, the uniform distribution of dystrophin beneath the plasma membrane has been interpreted as indicating that dystrophin has a similar role as part of the muscle membrane cytoskeleton^{4,13}. It may be speculated that in the brain dystrophin exists as a more or less uniformly distributed structural component. By contrast, the localization that we have demonstrated for dystrophin in neurons suggests a function more consistent with the anchoring of receptors or other elements of the postsynaptic apparatus at appropriate locations in the neuronal membrane. Such a role is in accord with the increase in dystrophin concentration at the nerve junctions in the electric organ of *Torpedo californica*¹⁴.

We have demonstrated here that the two brain regions richest in dystrophin are the cerebellar and the cerebral cortices. The cerebellum is involved in generating smooth coordinated movements and a defect in this structure might be expected to produce

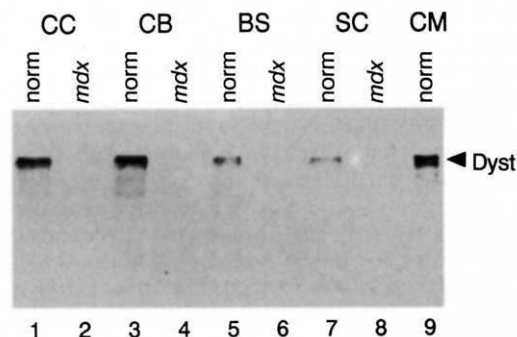


FIG. 1 Immunoblot analysis of dystrophin in muscle and brain tissue. Normal mouse, lanes 1, 3, 5, 7, 9; and *mdx* mouse, lanes 2, 4, 6, 8. Lanes 1 and 2, cerebral cortex (CC); lanes 3 and 4, cerebellum (CB); lanes 5 and 6, brain stem (BS); lanes 7 and 8, spinal cord (SC); lane 9, cardiac muscle (CM) (*mdx* showed no dystrophin staining). Lane 9 was loaded with 40-fold less tissue than the other lanes. The location of dystrophin is indicated; the doublet appearance is typical of mouse muscle⁷. Only the region between ~130K and the origin is shown.

METHODS. Tissues were homogenized in a buffer containing 10% SDS, 0.1 M Tris buffer (pH 8.0), 10 mM EDTA, 50 mM dithiothreitol, 0.25 mM PMSF, 25 μ M ml⁻¹ pepstatin, 100 μ M ml⁻¹ leupeptin and 1 mM iodoacetamide. About 300 μ g (wet weight) of tissue per lane was separated on a 0.75 mm thick, 7% acrylamide, 0.08% bis-acrylamide gel²⁰ followed by transfer to nitrocellulose. Blots were stained with 0.3 μ g ml⁻¹ affinity-purified antibody 6-10 in TBS plus 5% FCS and 0.1% Tween-20 at room temperature for 2 h. For preparation of antibody 6-10, dystrophin complementary DNA from residue 6,181-9,544 (ref. 11) was inserted into the pGEX-2T vector²¹ for expression of a dystrophin polypeptide fused to glutathione S-transferase. The fusion polypeptide was affinity-purified and cleaved with thrombin in accordance with the methods of Smith and Johnson²¹. The dystrophin polypeptide was purified and used to immunize a rabbit as in ref. 22. The antiserum was then affinity-purified by adsorption to a Hydropore EP column (Rainin Instruments) coupled with 2 mg purified fusion protein, followed by elution with 0.5 M glycine-chloride (pH 2.5) and dialysis into PBS.

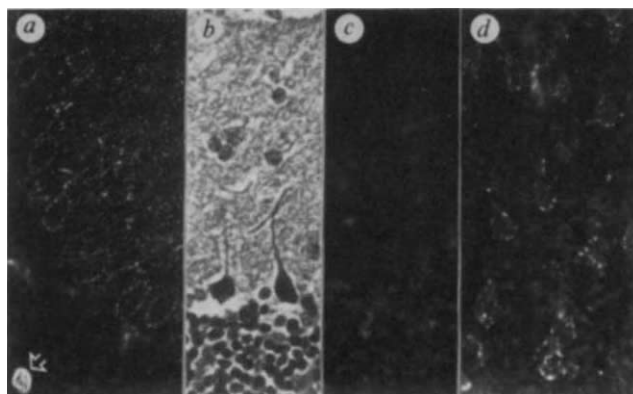


FIG. 2 Immunofluorescence localization of dystrophin. Cryosections (5-10 μ m) of: Normal mouse cerebellar cortex (a), *mdx* mouse cerebellar cortex (c) and normal mouse cerebral cortex (d) labelled with anti 6-10. Toluidine blue-stained sections of cerebellar cortex (b) provides cytoarchitectural reference. Note the intense punctate dystrophin immunoreactivity along the Purkinje cell bodies and dendrites (a) and the cerebral cortical pyramidal cell bodies and dendrites (d). The open arrow in a indicates a small artery; the wall is intensely reactive for dystrophin. No dystrophin immunoreactivity is present in the granule cell layer at the bottom of a or in *mdx* sections. Magnification: a-c, $\times 408$; d, $\times 624$.

METHODS. Mice were killed, and their brains dissected out and frozen in nitrogen-cooled isopentane. Frozen sections were cut, and indirect immunofluorescence was performed as described by Grzanna²³. Primary incubation, methanol-fixed frozen sections (7 μ m) with 0.3 μ g ml⁻¹ antibody 6-10 in PBS with 10% FCS, 1% Triton X-100, 4 $^{\circ}$ C overnight; secondary incubation, in DEAE column-purified TRITC-conjugated goat anti-rabbit IgG, 1:100, 90 min. Sections were examined with Zeiss Microphot epi-illuminated microscope.

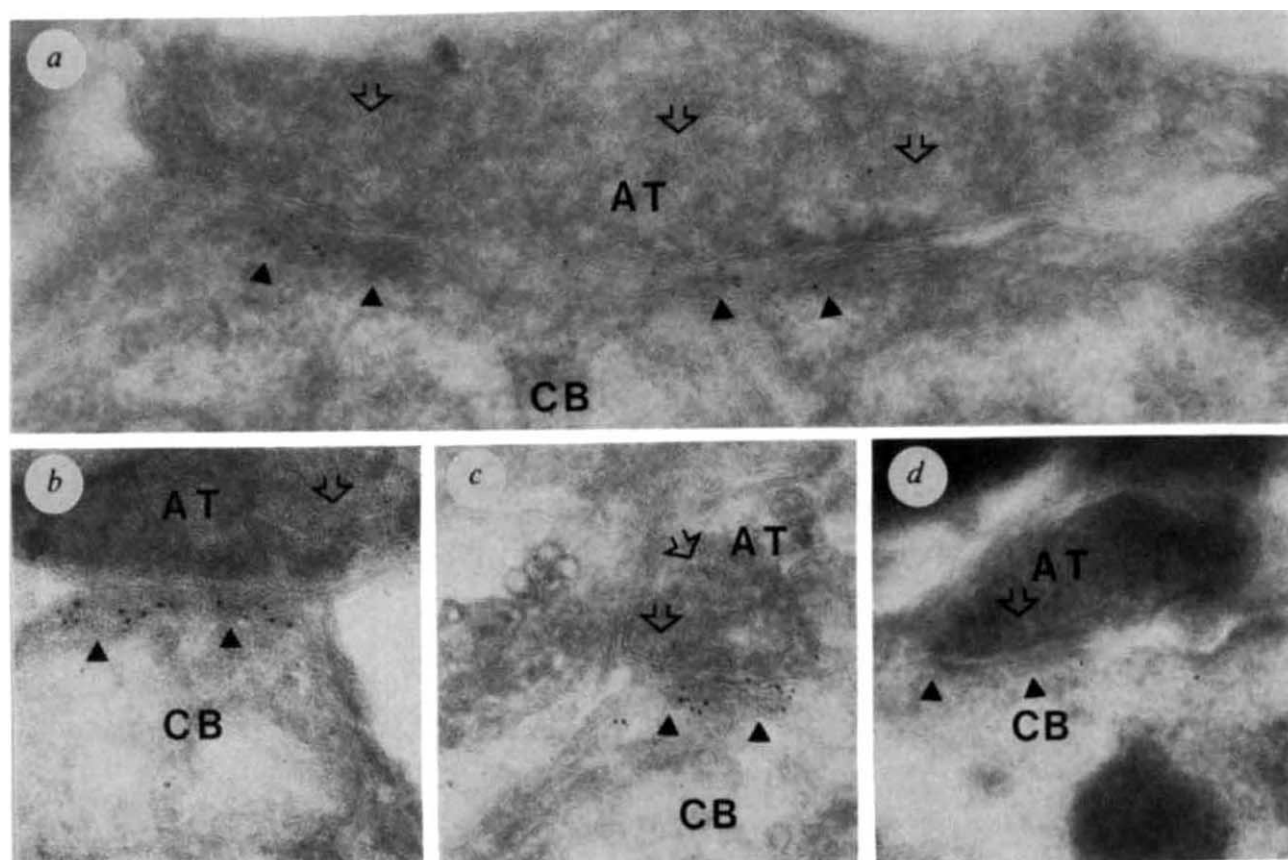


FIG. 3 Immunogold electron micrographs of synapses on Purkinje cell bodies. Ultrathin cryosections of normal mouse (a-c); *mdx* mouse (d) labelled with anti 6-10. Arrowheads indicate areas of postsynaptic membrane specializations. Open arrows show membrane-bound vesicles in the presynaptic profile. AT, axonal terminal (presynaptic); CB, cell body (postsynaptic). Magnification: a, $\times 94,000$; b, $\times 108,000$; c, $\times 87,000$; d, $\times 72,000$.

METHODS. Mice were killed and perfused with 2% paraformaldehyde, 0.01%

glutaraldehyde in PBS by intracardiac perfusion. Brains were removed, post-fixed in the same fixative for 1 h, rinsed with PBS, dissected and specimens prepared, sectioned and labelled as described previously²⁴. Primary antibody concentration was $5.2 \mu\text{g ml}^{-1}$ and was revealed by 5 nm goat anti-rabbit gold conjugate (Amersham). All other labelling and observation methods were as described elsewhere²⁵.

ataxia or tremor. In fact, in older *mdx* mice (12 months), tremors and mild incoordination are seen¹⁵, but these are not features of DMD, and the degree of pathology necessary to produce overt deficits is not known. The cerebral cortex is the brain region most strongly associated with cognitive function, and a defect in a major class of cerebral cortical neurons would be expected to produce a cognitive deficit. We have demonstrated the basis for such an alteration in the localization of dystrophin at the synapses of cortical neurons and its absence in *mdx* mouse. A defect that affected all neurons or all synapses would probably be devastating, so this localization of dystrophin to a subpopulation of neurons and subclass of synapses is in keeping with the moderate cognitive dysfunction that characterizes DMD. Given the limited distribution of dystrophin, it is not surprising that previous surveys of the CNS have not detected any overt morphological abnormality. A subtle defect in some cells and at some synapses, the presence of other functionally related molecules in the spectrin family, and the considerable capacity for compensation in the developing nervous system, may all contribute to the mildness and variability of the neurological deficits in DMD.

There are so far few mechanistic models for the defects that produce mental retardation. Several of the inborn errors of metabolism are thought to produce massive neuronal loss and such a profound and progressive loss of cognitive function that they are more appropriately termed dementias. Trisomy 21 has been exhaustively studied, and in this condition, showing more

moderate mental retardation, an abnormality in synaptic organization may be the underlying mechanism of cognitive dysfunction¹⁶⁻¹⁹. The identification of dystrophin at cerebral cortical synapses is in keeping with such a mechanism and the precise localization of the DMD gene product provides a focus for further analysis. On the basis of results reported here, together with the previous finding of mild incoordination and tremors in the *mdx* mouse¹⁵, it would seem justified both to re-examine clinical features of DMD that might better characterize the CNS dysfunction, and attempt to find alterations in synaptic structure and function that are the phenotypic basis of the cognitive defect. □

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Cell-autonomous action of zebrafish *spt-1* mutation in specific mesodermal precursors

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IN zebrafish, as in *Xenopus*¹, the well-orchestrated cell movements of gastrulation can be dissected into several components, including epiboly², involution^{3,4}, convergence and extension⁴. Embryos homozygous for the recessive lethal mutation *spt-1(b104)* or 'spadetail' have a complex set of defects in the trunk of the embryo that may arise secondarily after loss of one of these movements, convergence, from those precursors that would normally have given rise to trunk somitic mesoderm⁵ (Fig. 1). We have now tested this hypothesis by transplanting cells between wild-type and mutant embryos, to identify the cells that *spt-1* affects directly. Our results show that the mutation autonomously affects only those mesodermal precursors located along the lateral margin of the early gastrula blastoderm⁶. Other mesodermal cells and all ectodermal precursors seem not to require function of the wild-type gene. Our findings reveal an unexpectedly delicate genetic control of vertebrate gastrulation.

For each experiment, separate groups of cells from two distinctively labelled donor embryos were cotransplanted at the

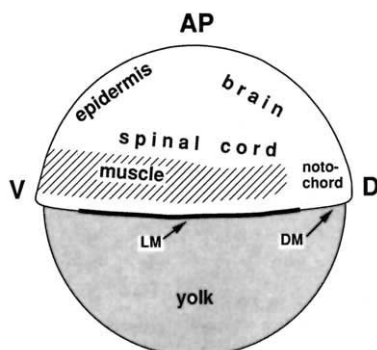


FIG. 1 Fate map (redrawn from ref. 6) of the zebrafish embryo at 50% epiboly, the onset of gastrulation (5.2 h). At this stage the blastoderm, in the form of a cup inverted atop the single large yolk cell, contains approximately 8,000 cells. The slashed region delineates the mesodermal precursors, which are in the lateral marginal zone, that are affected by the *spt-1* mutation. In wild-type embryos, these precursors involute at the lateral margin and converge dorsally to form the paraxial mesoderm, which generates somites. Later, the somites form myotomes, blocks of body muscle. The dorsal marginal zone involutes to form the axial mesoderm, primarily notochord. D, dorsal; V, ventral; AP, animal pole; LM, lateral margin; DM, dorsal margin.

late blastula stage into an unlabelled wild-type host embryo (Fig. 2) or, occasionally, into a *spt-1* host. This design permitted the direct and simultaneous comparisons of the movements and fates of wild-type and mutant cells beginning gastrulation together at exactly the same position. Combinations in which both donors were of the same genotype (that is wild type:wild type and *spt-1*:*spt-1*) served as controls.

When labelled wild-type and *spt-1* cells were cotransplanted into the lateral marginal zone of either wild-type or mutant host embryos, the labelled cells from wild-type donors converged dorsally in a normal fashion. In contrast, the cotransplanted *spt-1* cells failed to converge, even though they were placed in the same environment as the wild-type cells transplanted with them (Fig. 3a). The *spt-1* cells moved caudally during gastrulation and entered the tailbud that forms after epiboly. Thus, by this stage, the wild-type and mutant cells had segregated as

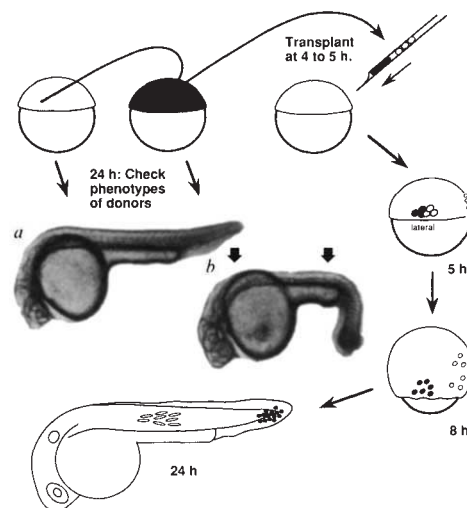


FIG. 2 Comparison of a, wild-type and b, *spt-1* zebrafish embryos at 24 h. The trunk area of the mutant, delimited by the small arrowheads, is grossly deficient in muscle cells and lacks the segmental chevron-shaped pattern of organized myotomes. Also, note the characteristically bent notochord and excess of cells at the end of the tail.

METHODS. Embryos for experiments were produced⁵ by crossing heterozygotes for *spt-1*, yielding wild-type and *spt-1* embryos in a 3:1 ratio. As the mutant phenotype is not visible before 12 h, the choice of donors and hosts for the transplantation procedures is done blindly. Donor embryos were labelled, some with rhodamine-dextran (RDA) and others with fluorescence-dextran (FDA, both dyes from Molecular Probes, 5 mg ml⁻¹ in 0.2 M KCl), by intracellular injection of dye into the yolk cell before the 16-cell stage (1.5 h). Hosts, sibs of the donors, were not labelled. Genotypes of donor embryos were inferred from their later appearing phenotypes. Pipettes used for transplantations were made on a Flaming-Brown puller using non-capillary electrode glass. Tips of pipettes were either bevelled or broken off to an inner diameter of 10–30 μm and polished on a microforge. Often, a sharp point was tooled onto the end of the pipette to aid penetration of outer tissue layers. Pipettes were filled with mineral oil and attached via polyethylene tubing to an oil-filled Hamilton syringe. Transplantation procedures were monitored using a Zeiss UEM microscope equipped with ultraviolet epilumination and Silicon Intensified Target video camera (Dage-Mit, Inc., Model SIT 66). Before gastrulation (5 h) a small group of 5–10 cells from one labelled embryo was gently drawn up into the transplantation pipette followed by another group of cells taken from a second, differently labelled donor embryo. Donor cells were collected from a mid anteroposterior position in the blastoderm but randomly with respect to dorsal-ventral position because dorsal-ventral polarity is not yet apparent. Both groups of cells were then simultaneously transplanted into an unlabelled host embryo. The locations of the labelled cells were recorded immediately after transplantation and again at 12, 24 and 48 h of development. At 12–14 h, the phenotypes of the donor embryos were recorded to establish the identities of the RDA- and FDA-labelled donor cells. Individual images were stored on a Panasonic optical disk recorder model TQ-3031F. Images were then superimposed and pseudocoloured using the imaging software 'NeuroVideo' running on a Mac II computer.

groups into very different locations within the embryo (Fig. 3b).

Wild-type cells transplanted into the lateral margin of hosts of either genotype later expressed a fate appropriate for the lateral marginal zone of wild-type embryos, that is, myotomal muscle in the trunk. The *spt-1* cells transplanted into the lateral margin of hosts of either genotype later expressed a fate appropriate for the lateral marginal zone of mutant embryos, namely, mesenchyme in the tail (Fig. 3c). In the three cases where wild-type and *spt-1* cells were transplanted into mutant hosts, the wild-type cells converged as expected, forming trunk muscle cells, and the mutant cells formed a number of derivatives in the tail.

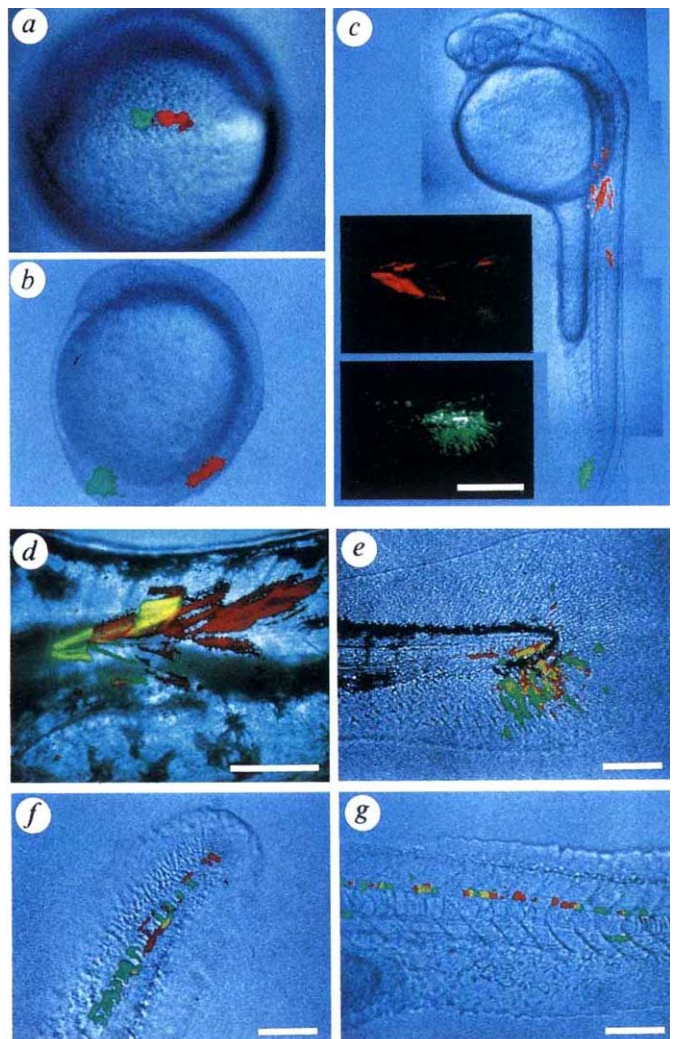
We interpret these results to mean that *spt-1* acts autonomously in the precursors of the trunk somitic mesoderm. In wild-type embryos, cells beginning gastrulation together never separate so completely with respect to both cell movement and eventual fate⁶. Also, separation of donor cells did not occur when the cells transplanted to the lateral marginal zone were from donors of the same genotype. In these controls, all the donor cells migrated together and their progeny were frequently intermingled. As expected, wild type cotransplants tended to express trunk myotomal muscle fates (Fig. 3d), and *spt-1:spt-1* cotransplants tended to express tail mesenchyme (Fig. 3e).

Is function of the wild-type gene (*spt-1*+) required in other gastrula cells? Changes in the early movements of other mesodermal precursors were not previously detected in *spt-1* mutants, although changes in some nonsomitic mesodermal derivatives are present later. One of these is the notochord, which fails to lengthen normally, becoming kinked and twisted⁷.

As in frogs⁷, the notochord in zebrafish forms from dorsal mesoderm by extension movements driven by cell intercalations, and it is possible that the notochordal defects in *spt-1* embryos result from a failure of normal extension movements. In our experiments, however, when wild-type and mutant cells were cotransplanted into the dorsal marginal zone, the transplanted cells of both genotypes intermingled with the unlabelled host cells in the notochord (Fig. 3f). As *spt-1* function is required for normal cell intercalations of paraxial mesoderm^{1,4}, this result indicates that the intercalations that produce extension of dorsal axial mesoderm occur independently of *spt-1* function, and that the kinking of the notochord in mutants must arise secondarily to its extension.

Ectodermal precursors lateral to the dorsal midline normally converge to form the spinal cord, just as mesodermal precursors converge to form the somites⁴. The spinal cord is present and approximately of normal size in the trunk of *spt-1* mutants, suggesting that the convergence in the ectoderm is normal. We tested this directly by transplanting the mixtures of cells to the lateral nonmarginal zone of the blastoderm, which generates the spinal cord (Fig. 1). We consistently observed that cotransplanted wild-type and mutant ectodermal precursors converged dorsally together, and remained mixed together during their movements that form the spinal cord, irrespective of the host genotype (Fig. 3g). We conclude that the *spt-1* product is not necessary for the proper convergence of ectodermal precursors. We suspect that the ectodermal irregularities seen in mutant embryos, that is mislocation of pigment cells and abnormal motoneuron axons, are secondary consequences of the lack of trunk somites in *spt-1* embryos.

FIG. 3 The *spt-1* mutation specifically and cell autonomously affected the movements of lateral marginal zone cells. **a**, Zebrafish embryo just before gastrulation (5 h) and immediately following cell transplantation. Two groups of cells from two different donor embryos were transplanted into the lateral marginal zone of a wild-type host embryo ($n=11$). The FDA-labelled cells (coloured green) came from an embryo mutant for *spt-1* and the RDA-labelled cells (red) came from a wild-type donor. **b**, Same embryo after gastrulation (12 h). The two groups of cells segregated into different areas of the animal; the RDA-labelled wild-type cells converged to the dorsal axis, whereas the mutant cells moved to the tailbud region. Diameter of the embryo shown in **a** and **b** is 500 μm . **c**, Same embryo at 30 h. The wild-type cells, in the trunk, were anterior to the *spt-1* cells, in the tail. Wild-type and *spt-1* donor cells showed the same general pattern in both wild-type ($n=11$) and *spt-1* hosts ($n=3$). We never observed the reverse, that is, *spt-1* cells anterior to wild-type cells (Fisher sign test, $P<0.001$). Length of the embryo in **c** is ~ 1.5 mm. Orientation for **a**, **b** and **c** is animal pole (or anterior) towards the top of the page and dorsal to the right. **c**, Upper inset, transplanted, RDA-labelled cells of the same embryo at 48 h. The transplanted wild-type cells gave rise to striated myotomal muscle cells. **c**, Lower inset, the tail region of the same embryo at 48 h showing that the transplanted, FDA-labelled *spt-1* cells gave rise to the fin rays and other associated mesenchymal derivatives. Orientation for the two insets is anterior to the left and dorsal towards the top of the page. Scale bar, 100 μm . **d**, Cotransplanted wild-type cells did not segregate. When cells from two wild-type embryos were transplanted into the lateral marginal zone of a wild-type host ($n=17$), the two groups of donor cells migrated together and the intermingled progeny cells differentiated into trunk myotomal muscle by 48 h. **e**, Cotransplanted mutant cells did not segregate. When both groups of donor cells were of the mutant *spt-1* genotype and transplanted to the lateral marginal zone of a wild-type host ($n=3$), both groups of cells took up positions within the tail where they formed predominately tail mesenchyme by 48 h. **f**, The *spt-1* mutation did not affect the movements of precursors at the dorsal margin. FDA-labelled mutant cells and RDA-labelled wild-type cells did not segregate apart when placed within the dorsal marginal zone ($n=3$). By 30 h both groups of cells gave rise to intermingled notochord cells along the length of the axis, including the tail region as shown here in a wild-type host. **g**, The *spt-1* mutation did not affect the movements of ectodermal precursors. FDA-labelled mutant cells and RDA-labelled wild-type cells migrated together and formed ectodermal derivatives when placed in the nonmarginal zone of a wild-type host ($n=25$). This figure shows the intermingling of floor plate cells in the spinal cord at 24 h. Similar results occurred with neuronal and epidermal precursors. Orientation of **d**, **e**, **f** and **g** is anterior to the left and dorsal towards the top of the page. Scale bars, 100 μm .



Using genetic mosaics, we have shown that the *spt-1* mutation seems exclusively to affect a single gastrulation movement of cells within the lateral marginal zone of the blastoderm. Furthermore, the mutation acts cell autonomously in these cells, as the placement of mutant cells into the lateral marginal zone of a wild-type embryo fails to rescue them into the wild-type pattern of migration. These findings relate domains of cellular movements and cell fate with an early, tissue-specific gene function: the *spt-1+* gene product seems to be necessary for the correct specification of prospective trunk somitic mesoderm whose precursor cells need to undergo convergence during gastrulation. Furthermore, our findings indicate that convergence of other precursors, such as ectodermal cells, may depend on different underlying mechanisms, because the *spt-1+* product seems not to be required in ectodermal precursors. Neither is it required for extension movements of dorsally located mesodermal precursors of the notochord. Further characterization of the *spt-1* mutation, as well as attempts to clone *spt-1+*, are now underway to help us understand how the gene perturbs cell rearrangement, and should reveal to what extent the domain of gene expression coincides with its functional boundaries as delineated by our transplantation results. □

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Cloning and expression of a cDNA encoding an endothelin receptor

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ENDOTHELINS are a newly described peptide family consisting of three peptides (ET-1, ET-2 and ET-3) which are the most potent vasoconstrictive peptides known^{1–3}. They are crucial in the regulation of vascular smooth muscle tone^{1–3}. The diverse functions of endothelins are thought to be mediated by interaction with many different receptors coupled to the inositol phosphate/calcium ion messenger pathway. However, because of the structural resemblance of the three peptides, the presence and nature of multiple endothelin receptors remain to be elucidated. We report here the cloning of a complementary DNA encoding a bovine endothelin receptor, which has a transmembrane topology similar to that of other G protein-coupled receptors and shows specific binding, with the highest selectivity to ET-1 in animal cells transfected with the cloned cDNA. This receptor messenger RNA is widely distributed in the central nervous system and peripheral tissues, particularly in the heart and lung. Our results support the view that there are other receptor subtypes.

We previously reported an expression-cloning strategy for receptors that can be electrophysiologically assayed in *Xenopus* oocytes after injection of appropriate exogenous mRNAs^{4–7}. Because oocytes injected with the bovine lung mRNA showed a potent and specific electrophysiological response to applica-

tion of endothelin, we adopted this strategy for the isolation of a functional cDNA clone encoding a bovine receptor. A bovine lung cDNA library was constructed in an RNA expression vector from an mRNA fraction giving potent receptor expression in *Xenopus* oocytes. A functional receptor cDNA clone was identified and purified from the cDNA library by testing for expression in oocytes after injection of the mRNAs synthesized *in vitro* from the serially subdivided cDNA mixture. The cDNA clone was verified to encode a functional receptor in the oocyte expression system. A potent electrophysiological response was induced by application of ET-1 or ET-2, whereas a moderate response was produced by ET-3, but not by any of the peptides unrelated to endothelin which we analysed (data not shown). Furthermore, the endothelin-evoking currents were fluctuating and long-lasting and were thus characteristic of the response mediated through activation of the inositol phosphate/Ca²⁺ second messenger pathway⁸.

Figure 1 shows the 3,216-nucleotide sequence of the cloned cDNA and the amino-acid sequence deduced for the endothelin receptor, which was assigned from the longest open reading frame of the cDNA. The nucleotide sequence surrounding the initiation codon agrees well with the consensus sequence⁹. The deduced amino-acid sequence of the receptor consists of 427 amino-acid residues with a relative molecular mass of 48,516. Hydropathicity and amino-acid homology analyses of the deduced sequence show the existence of seven hydrophobic segments and a significant sequence similarity with G protein-coupled receptors^{10,11}. The receptor probably has seven membrane-spanning domains with an extracellular N terminus and a cytoplasmic C terminus.

The endothelin receptor is not particularly closely related to other peptide receptors^{4–7} in terms of sequence similarity (23–26% identity in the core sequence covering transmembrane segments I–VII). It has a relatively long N terminus preceding transmembrane segment I, and this portion, as in lutropin-choriogonadotropin and other large hormone receptors^{12,13}, may be involved in binding a relatively large endothelin peptide. There are several potential sites for post-translational modification in the receptor: a possible site for cleavage of an N-terminal hydrophobic sequence as a signal peptide; two consensus sites for N-glycosylation¹⁴ (Asn 29 and Asn 62); six cysteine residues in the C terminus near transmembrane VII, one of which may be palmitoylated as in the β_2 -adrenergic receptor¹⁵; and several serine residues for regulatory phosphorylation in the cytoplasmic domains^{16,17}. Another interesting feature is the similarity of the sequence (residues 251–261) near transmembrane V with the consensus sequence of domain 9 of the serine/threonine protein kinase family¹⁸, but the relevance of this sequence similarity is unknown.

Previous pharmacological and ligand-binding studies suggested that there are several types of endothelin receptor in mammalian tissues^{1,19,20}. However, the precise characterization of the individual receptors has been hampered by the cross-reactivity of each receptor with other endothelins. We determined the affinities of the cloned receptor for endothelin peptides in monkey kidney COS cells after transfection of the cloned cDNA. Binding of ¹²⁵I-labelled ET-1 to membranes prepared from cells transfected with the receptor cDNA was saturable with a dissociation constant (K_d) of 0.18 nM (Fig. 2a). No such binding was detected on membranes prepared from untransfected cells or from cells transfected with the vector DNA alone. Displacement experiments of ¹²⁵I-labelled ET-1 binding indicated that ET-1 is the most potent inhibitor of radioligand binding, being about 10 times more effective than ET-2 (Fig. 2b) although these two peptides differ in only 2 out of 21 amino-acid residues¹. An endothelin-like venom toxin, sarafotoxin S6b, and ET-3 also displaced the radioligand binding, but these two peptides were much less effective than either ET-1 or ET-2. The values of the inhibition constant (K_i) for ET-1, ET-2, sarafotoxin S6b and ET-3, were calculated to be

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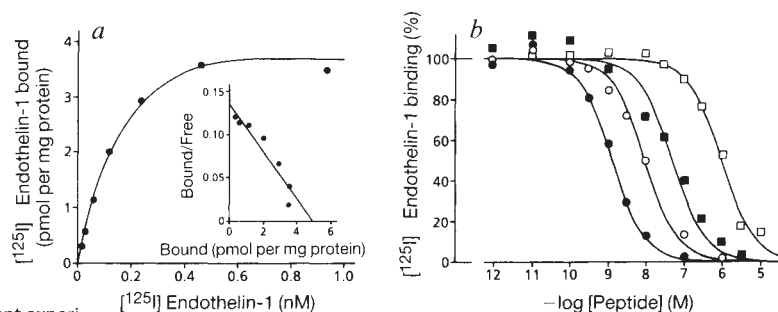
FIG. 1 The cDNA sequence for bovine endothelin receptor and its deduced amino-acid sequence. The deduced amino-acid sequence is shown above the cloned cDNA sequence. Positions of the putative transmembrane segments I-VII are indicated above the sequence; the termini of each segment are tentatively assigned on the basis of a hydrophaticity profile and sequence comparison with other G protein-coupled receptors. Triangles, potential *N*-glycosylation sites¹⁴; asterisks, amino-acid residues homologous to the consensus sequence of domain 9 of the Ser/Thr protein kinase family¹⁸; dots, possible phosphorylation sites^{16,17}. The possible polyadenylation signal²³ is underlined.

METHODS. Bovine lung poly(A)⁺ RNA was centrifuged on a sucrose density gradient⁵ (5–25%). The fraction giving potent receptor expression in *Xenopus* oocytes was identified by electrophysiological measurements and was used

for subsequent construction of a cDNA library with λ ZAPII DNA vector⁴⁻⁶. A portion of this library was divided into fractions, each containing $\sim 3 \times 10^4$ cDNA clones, and transcribed *in vitro* by T7 RNA polymerase in the presence of the capping nucleotide⁵. A fraction containing a functional receptor cDNA clone was identified by testing electrophysiological responses to application of ET-1 (100 nM) in oocytes injected with the mRNAs synthesized *in vitro*. A single cDNA clone was purified by repeating the *in vitro* mRNA synthesis and electrophysiological measurements after stepwise fractionation of the response-evoking cDNA mixture⁵. The λ ZAPII phage clone containing the endothelin receptor cDNA was converted to the plasmid cDNA (pBETRI) by rescue excision. The nucleotide sequence of both strands of the cDNA was determined by the chain-termination method²⁴.

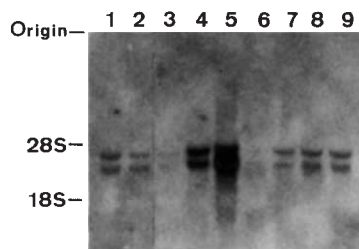
FIG. 2. *a*, Saturation isotherm and *b*, displacement of specific binding of ^{125}I -labelled ET-1 to membranes of cells transfected with receptor cDNA. *a*, Inset shows Scatchard plot of ^{125}I -labelled ET-1 binding; K_d and B_{max} were 0.18 nM and 4.9 pmol per mg protein, respectively. *b*, Binding of ^{125}I -labelled ET-1 was determined in the presence of the indicated concentrations of ET-1 (●), ET-2 (○), ET-3 (□) and sarafotoxin S6b (■).

METHODS. The *XhoI*-*NotI* fragment of pBETRI was subcloned into the eukaryotic expression vector CDM8. Transfection of the resultant receptor cDNA-CDM8 into COS cells and isolation of crude membranes of the cells transfected with DNA were as described previously⁷. Ligand binding was performed as described²⁵. [¹²⁵I]-labelled ET-1 (45 pM) was used for displacement experiments. Each experiment was carried out at least twice in triplicate, and the results shown here represent one experiment. ET-1 (1 μ M) was used to



define the nonspecific binding, and the specific binding activity amounted to >90% of the total binding activity.

FIG. 3 Northern blot analysis of RNAs isolated from various bovine tissues. Poly(A)⁺ RNA was isolated from the following tissues: 1, cerebral cortex; 2, midbrain; 3, adrenal gland; 4, lung; 5, heart; 6, liver; 7, kidney; 8, small intestine; 9, large intestine. RNA blot hybridization analysis (10 µg of RNA each) was carried out as described previously⁶ using the 959-bp *NcoI*-*EcoRI* fragment excised from clone pbETR1 as probe.



0.92, 7.2, 52 and 900 nM, respectively. The K_d and K_i values obtained for ET-1 agree well with those reported in binding studies of rat heart and brain membrane receptor²¹.

Northern blot analysis gave rise to two hybridizing bands with estimated mRNA sizes of ~3.3 and ~4.2 kilonucleotides (Fig. 3). The cloned cDNA corresponds to the smaller of the mRNAs. The larger mRNA is probably derived from the same receptor gene, because the band was seen when several cDNA fragments corresponding to different parts of the receptor mRNA were used for northern blot analysis under high-stringency conditions (data not shown). The northern analysis showed that the receptor mRNA is expressed in the highest amounts in the heart and lung, and is also present in the brain and many other peripheral tissues in lesser amounts.

Our pharmacological characterization demonstrates that the cloned cDNA encodes an ET-1 receptor. Many other classes of G protein-coupled receptors consist of multiple receptor subtypes which can be distinguished by their pharmacological properties and tissue distribution^{10,11,22}. Previous ligand-displacement experiments indicated an equal potency of ET-1 and ET-2 in inhibiting the binding of [¹²⁵I]-labelled ET-1 or ET-2 to chick cardiac membranes¹⁹. Furthermore, binding of [¹²⁵I]-labelled ET-3 to this membrane¹⁹ as well as to bovine endothelial cells²⁰ was more effectively displaced by ET-3 than by ET-1. These findings, taken together with the selectivity of the cloned receptor, support the view that additional receptor subtypes exist. Cloning of the ET-1 receptor cDNA will facilitate investigations of mechanisms of the function and regulation of this receptor and its possible role in pathophysiological processes, and also of the possible heterogeneity of the receptors. □

Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor

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ENDOTHELIN-1 was initially identified as a 21-residue potent vasoconstrictor peptide produced by vascular endothelial cells, but was subsequently found to have many effects on both vascular and non-vascular tissues^{1,2}. The discovery of three isopeptides of the endothelin family³, ET-1, ET-2 and ET-3, each possessing a diverse set of pharmacological activities of different potency, suggested the existence of several different endothelin receptor subtypes³⁻⁷. Endothelins may elicit biological responses by various signal-transduction mechanisms, including the G protein-coupled activation of phospholipase C and the activation of voltage-dependent Ca^{2+} channels⁸⁻¹⁰. Thus, different subtypes of the endothelin receptor may use different signal-transduction mechanisms. Here we report the cloning of a complementary DNA encoding one subtype belonging to the superfamily of G protein-coupled receptors. COS-7 cells transfected with the cDNA express specific and high-affinity binding sites for endothelins, responding to binding by the production of inositol phosphates and a transient increase in the concentration of intracellular free Ca^{2+} . The three endothelin isopeptides are roughly equipotent in displacing [¹²⁵I]-labelled ET-1 binding and causing Ca^{2+} mobilization. A messenger RNA corresponding to the cDNA is detected in many rat tissues including the brain, kidney and lung but not in vascular smooth muscle cells. These results indicate that this cDNA encodes a 'nonselective' subtype of the receptor which is different from the vascular smooth muscle receptor.

We constructed a cDNA library in the mammalian expression vector pCDM8 (ref. 11) from poly(A)⁺ RNA prepared from rat lung, which has abundant binding sites for endothelins. Sub-pools of the library were transfected into monolayers of COS-7 cells, which have no detectable receptor (Figs 1a and 2c). The transfected cells were screened for the expression of endothelin binding sites by incubation with [¹²⁵I]-labelled ET-1 followed by autoradiography (Fig. 1). This strategy identified a single recombinant clone, prETR-7, that confers ET-1 binding to COS-7 cells.

To determine the binding characteristics and functional properties of this receptor, we used COS-7 cells and COP-5 cells transiently transfected with prETR-7. The displacement of [¹²⁵I]-labelled ET-1 binding by unlabelled endothelin isopeptides in the transfected COP-5 cells (Fig. 2a) and COS-7 cells (data not shown) revealed that the expressed receptor has similar affinities for all three isopeptides. The transfected COS-7 cells responded to endothelin peptides with the production of inositol phosphates (Fig. 2b) and with a transient increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Fig. 2c). The dose-response relationships for $[Ca^{2+}]_i$ transients evoked by the three isopeptides were almost identical (Fig. 2d). The transfected COS-7 cells also responded to snake venom toxins, sarafotoxins S6b and S6c (ref. 5), with $[Ca^{2+}]_i$ transients (data not shown), confirming that sarafotoxins and endothelins share the same receptor.

Northern blots of poly(A)⁺ RNA from rat tissues were hybridized with the prETR-7 insert. A 5.0-kilobase (kb) receptor mRNA was detected in a number of rat tissues in which endothelins have been reported to have specific binding sites

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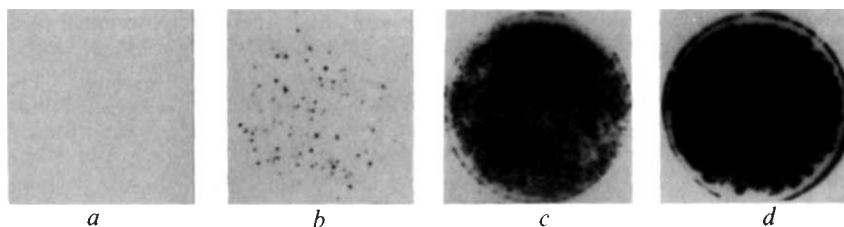
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FIG. 1 Autoradiograms for ^{125}I -labelled ET-1 bound to monolayers of COS-7 cells transfected with pCDM8 vector (a), a positive pool of ~500 individual cDNA clones, one of which encodes the ET_B receptor (primary screening) (b), a positive pool of 10 cDNA clones (secondary screening) (c), and a single cDNA clone prETR-7 (d).

METHODS. Total RNA was isolated from rat lung by the guanidine isothiocyanate/CsCl method²⁹ and poly(A)⁺ RNA was purified by chromatography on oligo(dT)-cellulose. Double-stranded cDNA was synthesized by a modified Gubler-Hoffman method²⁹, and ligated with non-paralindromic *Bst*XI linkers¹¹. The cDNA was size-fractionated (>1.8 kb) by gel-filtration chromatography on Sepharose CL-2B (Pharmacia), ligated to the mammalian expression vector pCDM8 (ref. 11), and introduced into *E. coli* Mc1061/P3. We obtained 5×10^5 independent colonies from 4 μg mRNA. Subconfluent monolayers of COS-7 cells grown in 60-mm culture dishes were transfected with 2 μg plasmid DNA from subpools of the library by the DEAE-dextran method with chloroquine treatment¹¹. Three days after transfection, monolayers were washed once with PBS and once with DMEM containing 0.3% BSA (binding buffer). Binding buffer (2 ml) containing $2.5 \times$



10^{-11} M 3-[^{125}I]iodotyrosyl¹³-labelled ET-1 ($2,000 \text{ Ci mmol}^{-1}$, Amersham) was added to each dish. After incubation at 37°C under humidified 5% $\text{CO}_2/95\%$ air for 2 h, the dishes were washed once with binding buffer and twice with PBS and fixed with 2.5% glutaraldehyde in PBS. The plates were air-dried, and, after the removal of the vertical edges, autoradiographed for 8–72 h at -80°C with intensifying screens. During the primary screening of the library with this procedure we detected 5 positive pools out of 2×10^4 individual cDNA clones; all these positive cDNA inserts could be hybridized to prETR-7 under conditions of high stringency (data not shown).

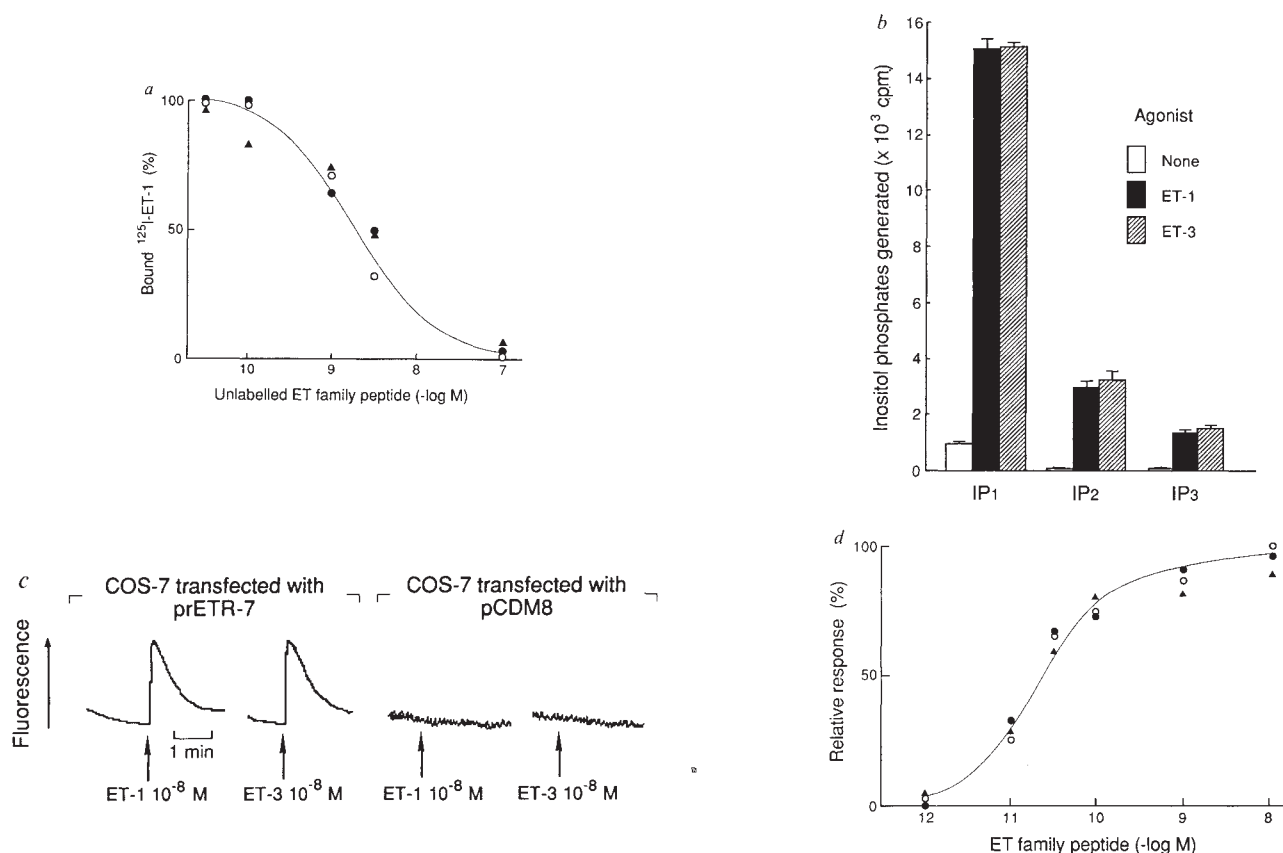


FIG. 2 Pharmacological characteristics of rat ET_B receptor exogenously expressed from prETR-7 in cultured mammalian cells. **a**, Displacement of ^{125}I -labelled ET-1 binding on prETR-7-transfected COP-5 cells by unlabeled ET-1 (●), ET-2 (○) and ET-3 (▲). The amount of bound ^{125}I -ET-1 without competitors in COP-5 cells transfected with pCDM8 vector was 4.8% of that observed in COP-5 cells transfected with prETR-7, and was indistinguishable from the level of nonspecific ^{125}I -labelled ET-1 binding in the presence of excess unlabelled ET-1. Each point represents the mean of duplicate determinations. **b**, Generation of inositol phosphates in prETR-7-transfected COS-7 cells in response to 10^{-7} M ET-1 or ET-3. Mean $\pm$ s.d. of triplicate determinations are shown. **c**, Typical examples for time-course of endothelin-induced changes in $[\text{Ca}^{2+}]_i$ in prETR-7-transfected COS-7 cells. **d**, Dose-response relationship of the amplitude of $[\text{Ca}^{2+}]_i$ transients elicited by ET-1 (●), ET-2 (○) and ET-3 (▲) in prETR-7 transfected COS-7 cells. Each point represents the mean of duplicate determinations of peak $[\text{Ca}^{2+}]_i$ increments expressed as a percentage of the value obtained with 10^{-8} M ET-1. **METHODS.** COP-5 cells were transfected with prETR-7 by the DEAE-dextran method, and the transfected cells were grown in 12-well plates. Three days after transfection, cells were washed twice with solution A (140 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4 , 1 mM MgCl_2 , 1.25 mM CaCl_2 , 11 mM glucose,

5 mM HEPES buffer, pH 7.4, 0.2% BSA). Cells were then incubated for 60 min at 37°C in 1 ml solution A containing 10^{-11} M ^{125}I -labelled ET-1 plus the indicated concentrations of unlabelled ET-1, ET-2 or ET-3 (Peptide Institute). After incubation, cells were washed extensively with solution A and the cell-bound radioactivity was determined. For functional assays, the transfected COS-7 cells were dispersed three days after transfection by 0.025% trypsin, 0.05% EDTA and trypsin action was terminated by adding soy-bean trypsin inhibitor. For determination of inositol phosphates, the cells were labelled with $10 \mu\text{Ci ml}^{-1}$ [^3H]myo-inositol (120 Ci mmol^{-1} , Amersham) in solution A for 3 h at 37°C , and stimulated with ET-1 or ET-3 for 30 min at 37°C in the presence of 10 mM LiCl. Inositol monophosphates, inositol bisphosphates and inositol trisphosphates were separated²³ by AG-1X8 anion exchange column chromatography (Bio-Rad) and the radioactivity was determined. For $[\text{Ca}^{2+}]_i$ transient assays, the collected cells were washed twice with solution A and incubated in solution A containing 4 μM Fura-2-penta-acetoxymethyl ester (Dojin) for 1 h at 20°C . The Fura-2-loaded cells were washed twice and resuspended in fresh solution A without the dye, and the fluorescence of the cells was measured²³ with excitation at 340 nm and 380 nm, and emission at 500 nm. $[\text{Ca}^{2+}]_i$ was estimated as described²³.

and/or pharmacological effects^{2,12}, suggesting that these activities may be partly attributed to the expression of this receptor (Fig. 3). Together with the observations that endothelin mRNAs are also expressed in many of these receptor-containing tissues¹³, the present findings suggest that endothelins are locally acting mediators. However, northern blot analysis of poly(A)⁺ RNA from rat aortas stripped of the endothelial and adventitial layers, as well as from rat aortic smooth muscle A-10 cells in culture, indicated that aortic smooth muscle cells do not express detectable levels of mRNA for this type of receptor (Fig. 3).

Figure 4 shows the nucleotide and deduced amino-acid sequence of the cDNA insert of prETR-7. The 3' noncoding region contains an AUUUA motif implicated in selective destabilization of mRNA¹⁴, suggesting that the receptor mRNA has a short intracellular half-life. The encoded polypeptide consists of 441 amino-acid residues beginning with a putative 26-residue signal sequence, as predicted by von Heijne's algorithm¹⁵. Thus the predicted mature peptide consists of 415 amino-acid residues with a calculated relative molecular mass (M_r) of 46,901. The difference between this value and those previously reported^{4,16} may be accounted for by glycosylation of the polypeptide (see below), possible proteolytic cleavage¹⁶, and/or the inaccuracy inherent in the estimation of the M_r of membrane proteins by SDS-PAGE. The encoded polypeptide contains seven stretches of 22–27 hydrophobic amino-acid residues, which are likely to represent transmembrane domains. The structure exhibits a significant sequence and topographical similarity with the photoreceptor rhodopsin and other G protein-coupled receptors. Amino-acid identities to other members of the superfamily are concentrated within the transmembrane domains (Fig. 4). The N-terminal region preceding the first putative transmembrane domain contains two potential N-glycosylation sites. The third cytoplasmic loop and the cytoplasmic C-terminal tail have several serine residues potentially able to be phosphorylated by serine/threonine protein kinases, suggesting that receptor function may be regulated by protein kinases. Other notable features of the endothelin receptor include the relatively long and proline-rich N-terminal portion, the short third cytoplasmic loop, the absence of an aspartate residue in the third transmembrane domain implicated in the binding of charged amine moieties in monoaminergic and muscarinic receptors¹⁷, and the

N-terminal signal sequence which is not found in G protein-coupled receptors, except the thyrotropin receptor¹⁸ and the luteinizing hormone/chorionic gonadotropin receptor¹⁹. The C-terminal residues 7 and 8 of the third cytoplasmic loop, which is one of the highly conserved regions in this superfamily, may be involved in the receptor/G protein interaction²⁰. However, this region in the endothelin receptor is not very similar to other members of the G protein-coupled receptor superfamily.

It has been previously shown *in vivo* that, whereas ET-1 and ET-2 are much more potent vasoconstrictors than ET-3, the three isopeptides are roughly equipotent in producing the transient vasodilatation^{3,7}, which may be evoked partly through the production of endothelium-derived relaxing factor²¹, sug-

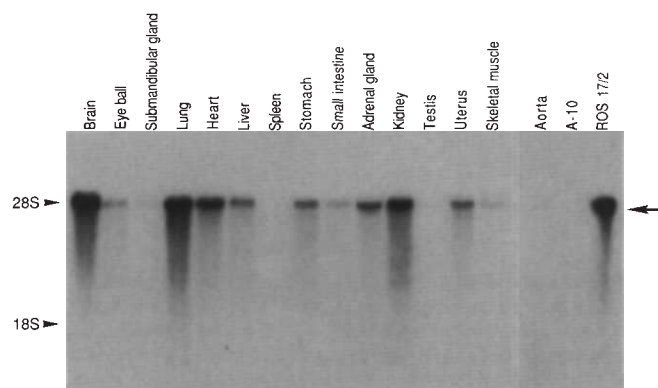


FIG. 3 Northern blot analysis of mRNA from rat tissues with prETR-7 insert as probe. The 5.0-kb E_{TB} receptor mRNA is indicated by an arrow. Aortas were carefully denuded of the endothelium and adventitia before RNA extraction. A-10, rat aortic smooth muscle A-10 cells; ROS 17/2, rat osteosarcoma ROS 17/2 cells. The presence of intact 28S ribosomal RNA was confirmed in all lanes (not shown).

METHODS. Poly(A)⁺ RNA prepared from rat tissues and cells as described in Fig. 1 was separated (10 μ g per lane) by formaldehyde/1.1% agarose gel electrophoresis, and transferred to a nylon membrane. The cDNA insert of prETR-7 was labelled to a specific activity of 8×10^8 c.p.m. μ g⁻¹ with [α -³²P]dCTP by the random priming method and used as probe. Hybridization was performed at 42 °C in 1 M NaCl, 50% formamide, 1% SDS, 250 μ g ml⁻¹ salmon sperm DNA. The membrane was washed in 0.1 $\times$ SSC, 0.1% SDS at 50 °C, and autoradiographed for 10 h at room temperature.

CGGGTGGCGTGGCCCAAGTTCCTCCATGGCCGCAAACTTAACCTACTGTG	-151
TGGCGGGGTAGAGACAACCGGTAGGTGAGTGTTCAGAGCGTGGCTGGTAGCTGACATAAGTACCTC	-76
CTTTCATATCCCGTGTGTCTCCAGACTGAAACCGGGGAGCGCTCTCAGAGGACCTCTCAGAGGACCTGCAAC	-1
ATGCAATCGTCCGCAAGCGGTGGCGACGCGCTTGGTGGCGCTGCTGGCTGTGGCTTGTGGGGTATGG	75
MetGlnSerSerAlaSerArgCysGlyArgAlaLeuValAlaLeuLeuLeuAlaCysGlyLeuLeuGlyValTrp	25
GGAGAGAAAGAGGATTCACCTGCCAGCCGACCATCTCTCTCGGGATTAAGAGTTATGACGCCACCC	150
GlyValAlaArgGlyPheProArgSerPheProArgSerGlyLeuLeuGlyValMetThrProPro	50
ACTAAGACCTCTGGACTAGAGGTTCACCTCCAGTCTGATGCGTTTCCGACTCGGAGGTGACCAAGGAGGG	225
ThrLysThrSerTrpThrArgGlySerAsnSerSerLeuMetArgPheArgThrAlaGluValThrLysGlyGly	75
AGGGTGGCTGGAGTCCCGCAAGATCTCTCCCTCTCGTCCCAAGCAAAATAGATCAACAAGCTTTTAA	300
ArgGluAlaGlyValProArgSerPheProArgSerGlyLeuLeuGlyValMetThrProPro	100
TATCATCAACAGATGTATCATGCTCGTGTCTGCTAGGCATCATCGGAGCTCCACACTGCTAAGAATCATC	375
TyrIleAsnThrIleValSerCysLeuValPheValLeuGlyIleIleGlyAsnSerThrLeuLeuArgIleIle	125
TACAAGAACAGTGCATGAGAAATGGTCCCAATATCTTGATCGCCAGCTGGCTCTGGGAGATCTGCACATC	450
TyrLysAsnLysCysMetArgAsnGlyProAsnIleLeuIleAlaGlyValLeuLeuGlyValLeuLeuHisIle	150
ATCATCGACATTCCTTAATGCTTACAAGCTGCTGGCAGGGGATGGCCATTTGGAGCTGAGATGGCAAGCTG	525
IleIleAspIleProIleAsnAlaTyrLysLeuLeuAlaGlyAspTrpPheGlyAlaGluMetCysLysLeu	175
GTGCCCTTCATACAGAGCTCTCTGGGGATCAGATGTTGAGTCTATGTGCTTAAGTATTCAGATATCGA	600
ValProPheIleGlnLysAlaSerValGlyIleThrValLeuSerLeuLeuGlyValLeuLeuSerLeuLeuArg	200
GCTGTGCTCTTGGAGTGAATTAAGGAATGGGGTCCAAATGGACAGCAGTAGAAATGTTTAAATTTGG	675
AlaValAlaSerTrpSerArgIleLysGlyIleGlyValProLysTrpThrAlaValGluIleValLeuIleTrp	225
GTGGTCTCTGTGTTCTGGCTGCTCCGTAAGCCATAGGTTTGTATGATACCTCGGACTCAAGGAAGGCC	750
ValValSerValValLeuAlaValPheGluAlaIleGlyPheAspValIleThrSerAspTyrLysGlyLysPro	250
CTAAGGGTCTGCATGCTTAATCCCTTCAGAAACAGCCTTCATGAGCTTTTACAAGACAGCCAAAGCTGGTGG	825
LeuArgValCysMetLeuAsnProPheGlnLysThrAlaPheMetGlnPheTyrLysThrAlaLysAspTrpTrp	275
CTGTTCAGTTCTACTCTGCTGCGCTAGCCATCACTGGCATCTTTACACCTTAAGACCTTGAGATGCTC	900
LeuPheSerPheThrPheCysLeuProLeuAlaIleThrValLeuSerLeuLeuGlyValLeuLeuSerLeuLeu	300
AGAAGAAAGTGGTATGAGATGCTTGAATGACCACTTAAAGCAGAGCAGAGAGTGGCCAGACAGTATTC	975
ArgLysLysSerGlyMetGlnIleAlaLeuAsnAspHisLeuLysGlnArgArgGluValAlaLysThrValPhe	325
TGCCTGCTCTGCTGTTTGGCTCTGTTGGCTTCCCTTCACCTCAGCAGGATTCGAAGCTCACCTTTATGAC	1050
CysLeuValLeuLeuPheAlaLeuCysTrpLeuProGluHisLeuSerArgPheGlyThrLeuLeuLysLeuLeu	350
CAGACATCTCAGAGTGTGAATCTCTGAGTTTGTGGTGTGGACTACATTTGGTATCAACATGGCTCT	1125
GlnSerAsnProGlnArgCysGluLeuLeuSerPheLeuLeuValLeuAspTyrIleGlyIleAsnMetAlaSer	375
TTGAATCTCGATTAATCCAATCGCTCTGTATTTGGTGAGCAAGAGATCAAAATCGTTTAAAGTCGTGTTG	1200
PheLysLeuLeuLeuLeuValSerLysArgPheLysCysPheLysCysPheLysCysLeuLeuLeuLeu	400
TGCTGCTGTGCCAAGCTTTGAGGAAACAGTCCCTAGAGGAGAGCAATCCTGCTGAAGTCAAGCTAAC	1275
CysCysTrpCysGlnThrPheGluGluLysGlnSerLeuGluGluLysGlnSerCysLeuLysPheLysAlaAsn	425
GATCAGGATACGACAACCTCCGCTCCAGCAATAATACAGCTCATCTTGAAGGAGGAGCACTCACTGAATCTC	1350
AspHisGlyTyrAspAsnPheArgSerSerAsnLysTyrSerSer***	441
ATTGTCTCATCTGGACAGATAGCATTAAACAAATGAAACCTTTGCCAACCCAAACGGAAACCGTGGT	1425
CGGAAAGGTGTGACGATGGGAGAGGATGTTTTTAAACCTTTCACTTTCCACACCTGATATTCACGGGC	1500
TTTACAACTAAGAAAGCCATGGGAATGAATGAAGCTCGGAAAGCACTTAGATCTTAGTCAAGACCTTCA	1575
GCACGGCTTTAAAGCCCTCACTGCACTCAGACCCACTTACATTTAAACCAAGACTCAACTCTATTCAAG	1650
AATTGCTGATTTGAGACAAAAACAAAAACAAAAA	1764

FIG. 4 Nucleotide and deduced amino-acid sequence of rat E_{TB} receptor cDNA cloned in prETR-7. The AUUUA sequence in the 3' nontranslated region, a selective mRNA destabilizing signal¹⁴, is underlined. An in-frame nonsense codon (nucleotides -48 to -46) is found upstream of the first ATG triplet which precedes the 1,323-bp open reading frame. The sequence surrounding this ATG agrees reasonably well with the favoured sequence that flanks functional initiation codons in eukaryotic mRNAs³⁰. The predicted 441-residue amino-acid sequence from this ATG is shown below the nucleotide sequence. The predicted cleavage site of the secretory signal sequence is indicated by an arrowhead. The putative transmembrane domains I–VII are underlined. Two potential N-glycosylation sites are indicated by arrows. Boxes show highly conserved amino-acid residues in the G protein-coupled receptor superfamily. Serine residues potentially able to be phosphorylated within the third cytoplasmic loop and the C-terminal tail are doubly underlined. **METHODS.** Restriction fragments of the cDNA insert of prETR-7 were subcloned into pUC118/119 phagemids and rescued as single-stranded DNA for sequence analysis. Both strands of overlapping subclones were sequenced by the dideoxy chain termination method with Sequenase T7 DNA polymerase (USB).

gesting that the receptors mediating these opposing responses are different. The present study unequivocally demonstrates the existence of a subtype of receptor that is nonselective among endothelin isopeptides. This pharmacological property is indeed different from that observed in the receptor(s) on vascular smooth muscle cells²² and several other types of cells^{23–26}, which to detect mRNA for the present endothelin receptor in vascular smooth muscle cells either *in vivo* or in culture. Receptors which do not distinguish between ET-1 and ET-3 have been identified pharmacologically in several cell types^{27,28}. Rat osteosarcoma ROS 17/2 cells, which respond equally to ET-1 and ET-3 with a transient increase of $[Ca^{2+}]_i$ (data not shown), express the present endothelin receptor mRNA in abundance (Fig. 3).

We propose that the non-isopeptide-selective endothelin receptor described in this study be called the ET_B endothelin receptor, whereas the ET-1/ET-2-selective subtype in, for example, vascular smooth muscle cells be called the ET_A receptor. Cloning of the ET_A receptor will enable us to understand better the mechanisms for the diverse physiological roles of the endothelin family. □

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Cytosolic Ca^{2+} gradients triggering unidirectional fluid secretion from exocrine pancreas

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EXOCRINE gland cells secrete Cl^- -rich fluid when stimulated by neurotransmitters or hormones¹. This is generally ascribed to a rise in cytosolic Ca^{2+} concentration² ($[Ca^{2+}]_i$), which leads to activation of Ca^{2+} -dependent ion channels^{3,4}. A precise understanding of Cl^- secretion from these cells has been hampered by a lack of knowledge about the spatial distribution of the Ca^{2+} signal and of the Ca^{2+} -dependent ion channels in the secreting epithelial cells⁴. We have now used the whole-cell patch-clamp method and digital imaging⁵ of $[Ca^{2+}]_i$ to examine the response of rat pancreatic acinar cells to acetylcholine. We found a polarization of $[Ca^{2+}]_i$ elevation and ion channel activation, and suggest that this comprises a novel 'push-pull' mechanism for unidirectional Cl^- secretion. This mechanism would represent a role for cytosolic Ca^{2+} gradients in cellular function. The cytosolic $[Ca^{2+}]_i$ gradients and oscillations of many other cells² could have similar roles.

Single acinar cells responded to acetylcholine (ACh) with a rise in $[Ca^{2+}]_i$ (Fig. 1)^{6–8}, which was mainly due to release of Ca^{2+} from internal stores⁹, because its amplitude was not altered by the removal of external Ca^{2+} (data not shown). Exposure to ACh also elicited an ionic current carried by Cl^- -permeable channels and an ionic current through cation-permeable channels^{10,11}. These currents were activated by an ACh-induced rise in $[Ca^{2+}]_i$ (refs 10–12), because they were eliminated by including the Ca^{2+} buffer, EGTA (data not shown).

The Cl^- current had two transient components ($n = 13$) at 0 mV, the reversal potential of the cation current^{10,11}. An early

component appeared abruptly, reached a peak within 0.5 s and then rapidly decayed (Fig. 1a–c); it occurred while there was little or no change in average $[Ca^{2+}]_i$ (Fig. 1a–c). The peak of the early component preceded the peak of the average $[Ca^{2+}]_i$ change by 1.5–8 s (mean 2.6 s, $n = 13$; Fig. 1). The early component was usually followed by a longer-lasting component (Fig. 1a, b), the peak amplitude and time of appearance of which were more variable than those of the early component (Fig. 1b). Both the time course and amplitude of the late component paralleled those of the average $[Ca^{2+}]_i$ signal (Fig. 1a–c). A similar biphasic Cl^- current occurs in the lacrimal gland¹³.

Current flowing through the cation-permeable channels, measured at –40 mV, the reversal potential of the Cl^- currents (E_{Cl})^{10,11}, was monophasic (Fig. 1d, middle trace; $n = 8$). The time course of the cation current correlated well with that of the rise in average $[Ca^{2+}]_i$ (Fig. 1d, lower trace). Thus, ACh first activates an early Cl^- current, which is followed by the simultaneous appearance of a cation current, a late Cl^- current and a rise in average $[Ca^{2+}]_i$ (Fig. 1d, upper trace).

That the early Cl^- current rises before the change in average $[Ca^{2+}]_i$ suggests spatial heterogeneity of the $[Ca^{2+}]_i$ signal^{8,14}. Digital imaging was used to determine whether spatial gradients in the $[Ca^{2+}]_i$ signal could explain this temporal discrepancy. In every cell examined ($n = 18$), ACh initially increased $[Ca^{2+}]_i$ at the edge of the luminal pole of the acinar cell and the $[Ca^{2+}]_i$ rise then slowly spread towards the basal pole (Fig. 2b–h). The same pattern of $[Ca^{2+}]_i$ rise was detected during Ca^{2+} oscillations induced by a lower concentration of ACh (0.33 μ M, data not shown). This pattern is surprising because the muscarinic receptors for ACh are thought to be localized to the basolateral membrane^{3,4}. This suggests that there is either positioning of ACh receptors close to the luminal membrane or luminal localization of specific Ca^{2+} -releasing organelles, highly sensitive to ACh stimulation, that have yet to be identified^{15,16}.

The spatial pattern of the ACh-induced Ca^{2+} signal is shown graphically in Fig. 3. The $[Ca^{2+}]_i$ signal was initially greatest at the luminal end and sharply declined in the basolateral direction, suggesting that Ca^{2+} release initially occurs just beneath the luminal plasma membrane. After the initial luminal rise in $[Ca^{2+}]_i$, a wave of elevated $[Ca^{2+}]_i$ spread towards the basolateral side and travelled at about 10 μ m s^{–1} (range 6–25 μ m s^{–1}, $n = 6$; ref. 2). During this Ca^{2+} wave, $[Ca^{2+}]_i$ continued to rise

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throughout the cell, usually reaching its maximal value at the basal pole (Fig. 3b, c). The spatial distribution of this secondary rise implies two different Ca^{2+} release mechanisms: a release from a store in the luminal side that is sensitive to inositol trisphosphate²; and a Ca^{2+} -activated Ca^{2+} release¹³ in the basolateral side. For comparison with the measurements of average $[\text{Ca}^{2+}]_i$ changes recorded during the patch-clamp experiments, a spatially averaged $[\text{Ca}^{2+}]_i$ signal was calculated from the imaging data (Fig. 3c, solid line). This signal lagged behind the luminal $[\text{Ca}^{2+}]_i$ changes by 0.1–0.9 s ($n = 5$). The initial luminal $[\text{Ca}^{2+}]_i$ rise was largely absent from the average $[\text{Ca}^{2+}]_i$ signal because of the small relative volume of the luminal pole.

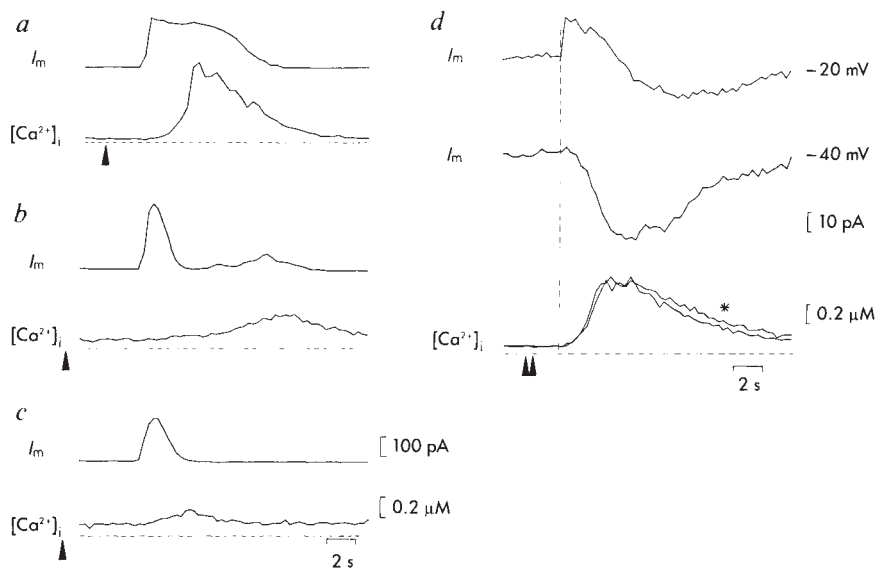
The spatio-temporal features of the Ca^{2+} signal allows interpretation of the pattern of activation of Ca^{2+} -dependent currents. The early Cl^- current is temporally correlated with the early spatially restricted increase in $[\text{Ca}^{2+}]_i$ at the luminal pole and, therefore, must flow through Cl^- channels in the luminal membrane. The peak amplitude of the early Cl^- current is always larger than that of the late Cl^- current (Fig. 1a–c), even though the relevant $[\text{Ca}^{2+}]_i$ levels and membrane areas are smaller at the luminal pole, suggesting that the Cl^- channels there are present at a high density and closely associated with Ca^{2+} -release

sites. The transient nature of the early Cl^- current could be caused by closure of the channel despite a sustained rise in luminal $[\text{Ca}^{2+}]_i$ (Fig. 3c) or could result from a highly localized decline in $[\text{Ca}^{2+}]_i$ that is beyond the spatial resolution of our measurements ($\sim 2 \mu\text{m}$). The late Cl^- current and the cation current could flow through the basolateral regions, because their activation most closely parallels the slower $[\text{Ca}^{2+}]_i$ changes in the bulk of the cell. This is consistent with previous findings that Cl^- (ref. 17) and cation¹⁸ conductances in the basolateral membrane of various exocrine cells are increased by ACh.

This pattern of channel activation suggests a 'push-pull' model for unidirectional Cl^- transport across the acinus (Fig. 4). In this model, secretagogues shift the acinar cell from a resting phase to a 'push' phase during which only the luminal Cl^- channels are activated. This will yield a secretion (or pushing) of Cl^- into the lumen because the membrane potential, V_m , is more negative than E_{Cl} (Fig. 4 legend). As the Ca^{2+} signal spreads, the 'pull' phase will occur because of the activation of the cation channel, which depolarizes V_m and reverses the electrochemical gradient for Cl^- . Chloride is then pulled into the acinus by influx through basolateral Cl^- channels. Given these fluxes of Cl^- , Na^+ may then flow through paracellular

FIG. 1 Average $[\text{Ca}^{2+}]_i$ and current responses to ACh ($10 \mu\text{M}$) recorded from single rat pancreatic acinar cells with combined Fura-2 and patch clamp measurements; a–d were recorded from four different cells. a–c, Membrane potential was held at 0 mV. ACh application was begun at times indicated by the arrowheads. d, Responses to two applications of ACh were measured at two different potentials (-20 and -40 mV); current traces are aligned to superimpose the $[\text{Ca}^{2+}]_i$ traces. Asterisk and left arrowhead refer to recordings made while the cell was held at -20 mV. Horizontal dotted lines indicate $[\text{Ca}^{2+}]_i$ of $0 \mu\text{M}$. A vertical dotted line in d shows the onset of the Cl^- current. Similar patterns of current activation and $[\text{Ca}^{2+}]_i$ rise were also seen with $1 \mu\text{M}$ and $0.5 \mu\text{M}$ ACh.

METHODS. Cells were dissociated from pancreatic acini of 3–6-week-old Wistar rats, using collagenase and trypsin¹⁰. A recording chamber was superfused with a solution containing: 140 mM NaCl, 2.8 mM KCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 11 mM glucose and 10 mM NaHEPES at pH 7.2. Patch pipettes (4–6 M Ω) were filled with a solution containing: 135 mM potassium glutamate, 20 mM NaCl, 1 mM MgCl_2 , 0.2 mM $\text{Na}_2\text{-ATP}$, 0.1 Fura-2 pentapotassium salt (Molecular Probes) and 10 mM Na-HEPES at pH 7.2. All measurements of potential were corrected for a junction potential between the pipette and bathing solution of -8 mV. Series resistance between the cells and pipettes ranged from 8 to 15 M Ω . Voltage



clamp experiments were made using an EPC-9 amplifier (HEKA Electronics). The methods used for measuring average $[\text{Ca}^{2+}]_i$ with a photomultiplier have been described²³. All experiments were at room temperature (23 – 26°C).

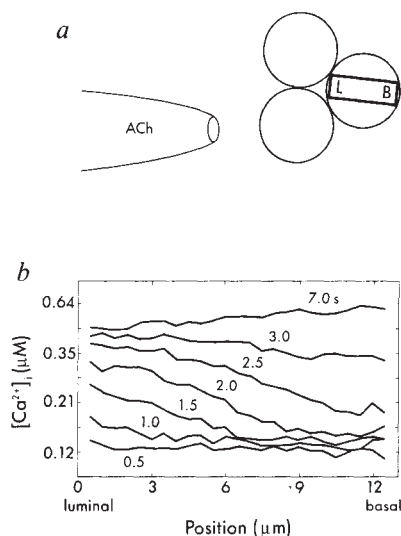


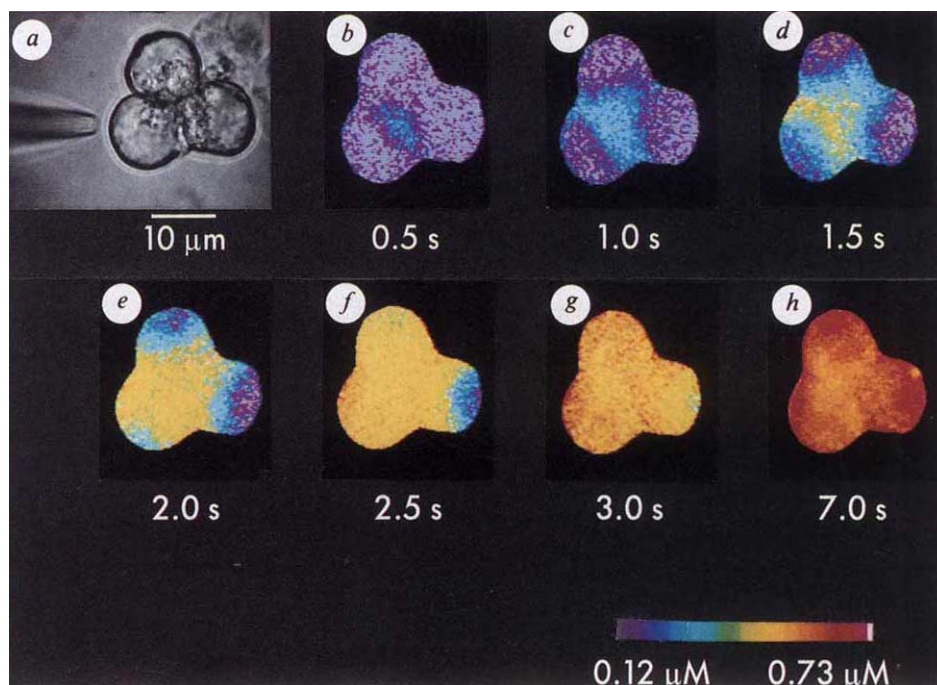
FIG. 3 Spatial and temporal features of ACh-induced $[\text{Ca}^{2+}]_i$ changes. a, Schematic diagram of the cell cluster shown in Fig. 2, indicating the scanning areas analysed in b and c. b, Spatial profile of $[\text{Ca}^{2+}]_i$ changes was scanned along the luminal-basal axis of the indicated cell. c, The time course of $[\text{Ca}^{2+}]_i$ changes caused by ACh were measured in $1\text{-}\mu\text{m}$ -wide areas at the two extreme poles of the cell (B and L in Fig. 3a). Solid line indicates the average change calculated by integrating over the entire area shown in a. Similar patterns were observed in all 18 cells tested, including three solitary cells not attached to a cluster. All cells within a cluster (or elsewhere within the field of ACh application) responded in this way, although the cells adjacent to the ACh-application pipettes invariably responded first (as in Fig. 2b).

pathways¹, because of the negativity of the lumen caused by the opening of luminal Cl^- channels³. Water flux will then osmotically follow these ion movements. Thus, the model proposes that the unidirectional secretion of iso-osmotic NaCl-rich fluid is directly caused by polarized activation of Ca^{2+} -dependent ion channels. Pumps and carriers^{3,4} have an indirect role, that is in generating the electrochemical gradients used by the ion channels during secretion. The model could represent the first example of a cellular function that requires cytosolic Ca^{2+} gradients. Minor variations in this model would account for Ca^{2+} -dependent fluid secretion from other exocrine glands^{1,3,4}

FIG. 2 Digital imaging of ACh-induced $[\text{Ca}^{2+}]_i$ changes in acinar cells. **a**, Bright-field micrograph of a cluster of three acinar cells. In the intact exocrine gland, part of the surface membrane of these cells (the luminal pole) is in contact with the lumen of the gland and the rest of it is in contact with the interstitial fluid (the basolateral membrane)². Even after cell dissociation, the presence of visible zymogen granules makes it easy to identify the luminal pole of the pancreatic acinar cell²⁶. Further, the attachments between cells within a triplet occurred at this pole. Thus, there was no ambiguity in defining cell orientation or the relative position of $[\text{Ca}^{2+}]_i$ changes within the cells. An application pipette containing 10 μM ACh is visible at the left of the cluster. **b–h**, Digital maps of changes in $[\text{Ca}^{2+}]_i$ induced by ACh. Timescale is relative to the earliest detectable response in the leftmost cell of the cluster. The changes in $[\text{Ca}^{2+}]_i$ were encoded with the pseudocolour scale shown at the bottom.

METHODS. Acinar cells were loaded with membrane-permeant Fura-2-AM, by incubating the cells with 3.3 μM Fura-2-AM for 30 min at 37 °C, and washed for 10 min at the same temperature. Details

of the methods used for high time-resolution digital imaging of Ca^{2+} are described in detail elsewhere²⁴. In brief, cells were excited with a single wavelength (390 nm, 10 nm bandpass) and the time-dependent changes in dye fluorescence were measured and digitally converted into a ratio by dividing by the fluorescence measured before agonist stimulation. As long as the path length and dye concentration are constant during the response, such a ratio yields a measure of relative $[\text{Ca}^{2+}]_i$, that is comparable to the more conventional two-excitation wavelength method⁵. This relative ratio can be converted to an absolute $[\text{Ca}^{2+}]_i$ level if the $[\text{Ca}^{2+}]_i$ before treatment is known (G.J.A. and E. Neher, manuscript in preparation). In our experiments, we assumed that the resting $[\text{Ca}^{2+}]_i$ was spatially uniform and was



(Fig. 4 legend).

The push-pull model circumvents previous difficulties in accounting for unidirectional fluid flow^{3,4} despite a predicted influx of Cl^- from the lumen to the acinus when the cation channels and the luminal Cl^- channels are simultaneously opened. Several observations support predictions that arise from the model. First, reducing the driving force for the inward movement of Cl^- during the pull phase should prevent secretion. Indeed, reducing external Na^+ concentration prevents ACh-dependent depolarization and inhibits secretion¹⁹. Second, reducing the driving force for the outward movement of Cl^-

equivalent to 0.12 μM , the mean of 12 two-wavelength measurements made with a photomultiplier (Fig. 1 legend). Fluorescence images were obtained with a SIT camera (effective frequency response ~ 10 Hz) and stored at video rates (30 images s^{-1}) on an optical disc video recorder (Panasonic TQ-2026F). Individual images were then digitized offline, with a Matrox MVP-AT frame grabber board, to calculate the fura-2 fluorescence change ratios. (Kanno *et al.*²⁵ have reported lower-resolution (0.033 Hz) two-wavelength ratio images from pancreatic cells treated with cholecystokinin. They have also found that this agonist produces sustained rises in $[\text{Ca}^{2+}]_i$ in the basolateral pole of these cells, but their measurements were too slow to capture the early luminal signal we report here.)

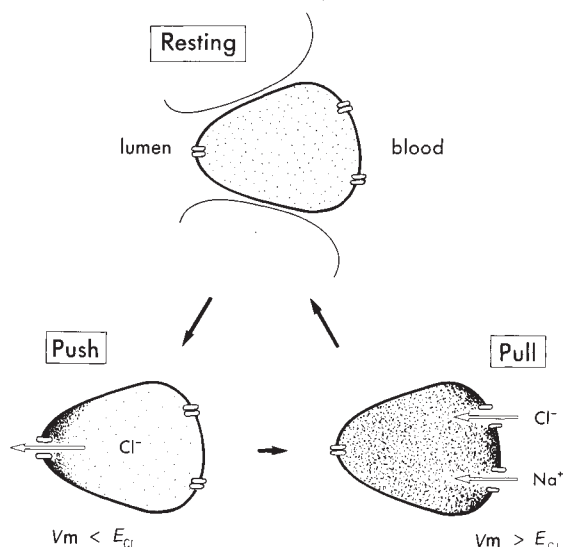


FIG. 4 A 'push-pull' model for Cl^- secretion in the rat pancreas. $[\text{Ca}^{2+}]_i$ is represented by the density of dotting. During a $[\text{Ca}^{2+}]_i$ rise induced by ACh, the acinus cycles through 'resting', 'push' and 'pull' phases. During the push phase, $[\text{Ca}^{2+}]_i$ selectively rises at the luminal pole of the cell and activates Cl^- channels. For Cl^- secretion into the lumen, V_m must be more negative than E_{Cl} at the luminal membrane. Microelectrode measurements in intact acini suggest that this is the case, because of a permeability of the resting cell to K^+ and active accumulation of Cl^- ($V_m = -39$ mV and $E_{\text{Cl}} = -33$ mV, ref. 1, assuming luminal fluid to be isotonic NaCl and isopotential to interstitial fluid). During the pull phase, the $[\text{Ca}^{2+}]_i$ increase spreads to the basolateral side to activate Cl^- and cation channels. For Cl^- uptake into the acinus, V_m must be more positive than E_{Cl} at the basolateral membrane. Measurements of the changes in V_m produced by ACh indicate that this is the case ($V_m = -20$ mV and $E_{\text{Cl}} = -33$ mV)⁴. Accumulation of internal Cl^- during the pull phase may also make E_{Cl} more positive and thereby enhance Cl^- secretion during the next push phase. The acinar cell may revert directly from the push phase to the resting phase, if a $[\text{Ca}^{2+}]_i$ rise is not sufficient to activate basolateral channels (as in Fig. 1c; see also Fig. 2 in ref. 8). Minor variations in this model also may account for Ca^{2+} -dependent fluid secretion from other exocrine glands^{1,3,4}; for example, adding Ca^{2+} -activated K^+ channels^{4,17} to the luminal pole could account for K^+ secretion from rat parotid gland.

during the push phase should prevent secretion; depolarization with high extracellular K^+ inhibits secretion²⁰. Finally, oscillatory $[Ca^{2+}]_i$ changes should yield more secretion than a sustained rise in $[Ca^{2+}]_i$; maximal secretion occurs at a concentration of ACh (0.3 μ M)²¹ which produces oscillations in $[Ca^{2+}]_i$ ^{8,9}. In blowfly salivary gland, secretion also increases with the frequency of V_m oscillations, presumably reflecting oscillatory $[Ca^{2+}]_i$ changes²². The generation and dissipation of localized $[Ca^{2+}]_i$ gradients may provide a rationale for the oscillatory $[Ca^{2+}]_i$ signalling of many cell types². □

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MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters

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THE T-cell immune response is directed against antigenic peptide fragments generated in intracellular compartments, the cytosol or the endocytic system¹. Peptides derived from cytosolic proteins, usually of biosynthetic origin, are presented efficiently to T-cell receptors by major histocompatibility complex (MHC) class I molecules^{2–4}, with which they assemble, probably in the endoplasmic reticulum (ER)⁵. In the absence of recognizable N-terminal signal sequences, such cytosolic peptides must be translocated across the ER membrane by a novel mechanism. Genes apparently involved in the normal assembly and transport of class I molecules may themselves be encoded in the MHC^{6–8}. Here we show that one of these, the rat *cim* gene⁶, maps to a highly polymorphic part

of the MHC class II region encoding two novel members of the family of transmembrane transporters related to multidrug resistance^{9,10}. Other members of this family of transporter proteins are known to be capable of transporting proteins^{11,12} and peptides¹³ across membranes independently of the classical secretory pathway. Such molecules are credible candidates for peptide pumps that move fragments of antigenic proteins from the cytosol into the ER^{14,15}.

The existence of a distinct peptide transporter for class I molecules was proposed initially to account for the properties of the mutant mouse cell line, RMA-S (ref. 5). Class I MHC molecules in RMA-S fail to assemble normally in the ER, and the cell is unable to present peptides of biosynthetic origin to T cells. The human cell line CEM.174 shows a similar phenotype, and in this case the lesion has been mapped to a large deletion in the MHC between DPA and the class III region¹⁶ (Fig. 1a). Natural alleles at the rat *cim* locus modify the specificity of class I antigen presentation and the time of residence of newly synthesized class I molecules in the ER (ref. 6, and S. Powis *et al.*, manuscript submitted). It has therefore been suggested that the *cim* locus product may also be involved in peptide loading or assembly of class I histocompatibility antigens. The *cim* locus has been mapped into the rat MHC as *RT1* (Fig. 1b) between the presumed DP homologue, *RT1.H* (represented by Pb in *H-2*, Fig. 1c), and the DQA homologue, *RT1.B₂* (represented by Aa in *H-2*) (A. M. Livingstone *et al.*, manuscript submitted). The enclosed segment is included in, but smaller than, the HLA deletion in the CEM.174 cell line.

We looked for *cim* by screening a rat concanavalin-A lymphoblast complementary DNA library¹⁷ using as probes inserts from an overlapping set of mouse cosmids covering the *cim* region¹⁸ (Fig. 1c). Two relatively abundant cDNAs (about 1 in 10⁴ clones) hybridizing with cosmids 5.10 and 508 were recovered. We propose the names *mtp1* and *mtp2* (for MHC-linked transporter protein) for the rat genes (homologous mouse cDNAs have recently been isolated, and the mouse genes named *HAM1* and *HAM2*¹⁹). The rat *mtp* genes were distantly related to each other because their cDNAs cross-hybridized weakly. The two genes were mapped into the known restriction maps of the mouse cosmids¹⁸ using rat cDNAs as probes (Fig. 1d). The mouse genes map 7 kilobases (kb) apart, about 14 kb centromeric to the Ob (formerly *A_{B2}*) gene. A search for rat cosmids containing the *mtp* region was unsuccessful; cloning difficulties in the homologous region in the rat MHC have been noted before²⁰. Accordingly, the rat *mtp* genes were mapped by restriction fragment length polymorphisms in Southern blots of genomic DNA from MHC congenic and recombinant rat strains (Fig. 2). Both genes mapped into the same region of the MHC as the *cim* gene. The genomic environments of both genes were notably polymorphic; three of the four genomic *Bam*H1 fragments of the *mtp1* gene showed allelic variation among the five haplotypes tested (Fig. 2a) as did the *Eco*R1 fragments of *mtp1*. The genomic blots showed no evidence for a family of close relatives.

The sequence of one putatively full-length *mtp1* cDNA clone, 510-15, is shown in Fig. 3. The 2,674-base-pair (bp) insert contains a single long open reading frame that is predicted to encode a polypeptide of 725 amino acids and to have a relative molecular mass of 79,000. The N terminus of the protein is conjectural, however spleen *mtp1* mRNA was indistinguishable in size by northern analysis from a T7 transcript of the 510-15 cDNA insert (data not shown). The *mtp1* polypeptide showed a highly significant sequence similarity to the ATP-dependent transmembrane transporter proteins of the multidrug resistance (*mdr*) group⁹, including both mammalian (*mdr*²¹, CFTR²²), yeast (STE6¹³) and prokaryotic (HlyB^{11,12}) members (Fig. 4a). Proteins of this family are active transporters for a wide range of substances including long polypeptides^{11,12}, oligopeptides¹³ and small hydrophobic compounds such as antibiotics and possibly steroids²³. The family displays a conservative cytosolic

C-terminal domain of about 250 amino acids with a characteristic series of motifs containing a consensus nucleotide-binding site²⁴ (the ATP-binding cassette, ABC²⁵) which it shares with members of a large prokaryotic protein family, most of which are also transport proteins²⁶⁻²⁸. In the *mdr* group of ABC pro-

teins, the ABC is appended to an N-terminal domain containing several strongly hydrophobic membrane-spanning sequences⁹. In many of the eukaryotic transporters this basic unit is duplicated in a tandem repeat. Like the prokaryotic polypeptide toxin transporters, HlyB and CyaB, the *mtp1* polypeptide consists of a single unit.

Although the hydrophobic segments of *mdr*-related transporters are more divergent than the ABC segment in amino-acid sequence, an apparent structural relationship is suggested by aligned hydrophobicity plots (Fig. 4b). In addition, *mtp1* shares with the single-unit bacterial transporters an N-terminal extension of about 100 amino acids. Interestingly, the predicted N terminus of *mtp1* has a hydrophobic sequence consistent with a typical signal peptide²⁹. The other mammalian transporters have strongly hydrophilic N termini which have been assumed to be cytoplasmic²¹. The two N-glycosylation signal sequences in the N-terminal half of *mtp1* are in a segment that would be available in the lumen of the ER if the membrane topology of HlyB^{9,30} (indicated in Fig. 4b) is conserved in this mammalian protein. A third potential N-glycosylation site at residue 517 is in the putative nucleotide-binding site and is presumably unused.

Figure 3 also indicates the sequence of a second full-length *mtp1* cDNA clone, 510-18, which showed a 171-nucleotide deletion (underlined) resulting in an in-frame deletion of 57

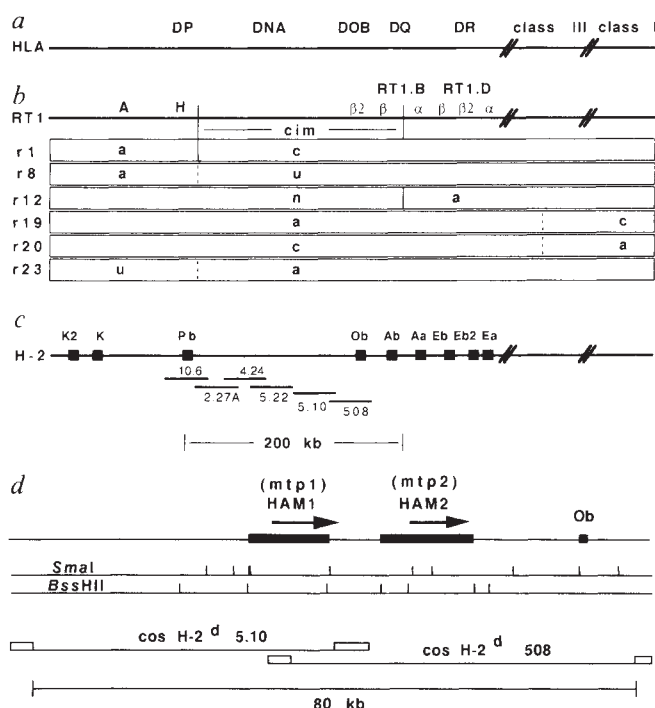
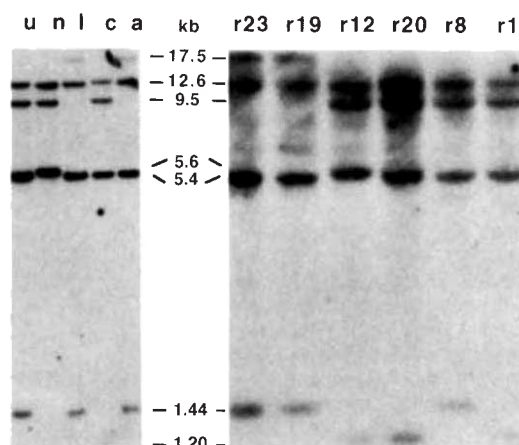


FIG. 1 Maps of the human (HLA), rat (RT1) and mouse (H-2) MHC complexes showing the general colinearity of homologous genes in the class II region. *a*, HLA system, not to scale, with the five principal class II gene families. No murine homologue of the DNA gene has so far been identified. *b*, Rat MHC, RT1, drawn to the same scale as H-2. The RT1.B and RT1.D class II genes are closely colinear with the H-2 homologues²⁰. The RT1.H genes have been identified as DP homologues in Southern blots^{34,35}. The interval between RT1.H and RT1.B_α contains the *cim* gene (A. M. Livingstone *et al.*, manuscript submitted). The distance between these two flanking markers has been estimated to be <320 kb by pulse-field gel electrophoresis (C. Carter, personal communication). The haplotype compositions of the six intra-MHC recombinant chromosomes (r1 to r23) have been described elsewhere³⁶ (A. M. Livingstone *et al.*, manuscript submitted). *c*, H-2 class II region drawn to scale from the overlapping cosmid map obtained by Steinmetz *et al.*¹⁸. The revised nomenclature of mouse class II genes is according to Klein *et al.*³⁷. Cosmids covering the interval of H-2 homologous to that containing the *cim* gene in RT1 are indicated. *d*, Location of the HAM1 (*mtp1*) and HAM2 (*mtp2*) genes defined by rat cDNA clones on the maps of the overlapping mouse cosmids 5.10 and 508. Arrows indicate 5' to 3' orientation. The names HAM1 and HAM2 are proposed in an independent analysis in the mouse¹⁹; the names *mtp1* and *mtp2* identify the rat homologues. The maps in Steinmetz *et al.*¹⁸ were confirmed, with the exception of an additional *SmaI* site enclosing a 500-bp fragment at the 5' end of the HAM1 (*mtp1*) gene.

METHODS. The vector arms of BALB/c (H-2^d) mouse cosmids¹⁸ 10.6, 4.24, 5.22 and 5.10 were removed by digestion with *NruI*. The inserts were then labelled by random priming and used as probes to identify homologous sequences in a λgt10 cDNA library prepared from lymphoblasts activated by concanavalin A (Con A), from a DA (RT1^a, *cim*^a) rat¹⁷. Hybridization to repetitive sequences was blocked by including 100 μg ml⁻¹ genomic DNA in the hybridization solution. A family of strong signals identified with the insert from cosmid 5.10 represented the rat *mtp1* gene. The rat *mtp2* gene was identified as a family of weaker signals from the 5.10 insert screen that hybridized strongly to a cDNA fragment of the mouse HAM2 gene¹⁹. Ten cDNA inserts from the *mtp1* series were subcloned into the *EcoRI* site of pBluescript KS(+) and end-sequenced, and four putatively full-length clones identified. One of these, 510-15, was subsequently used to probe restriction digests of the cosmids in the Pb to Ob interval. A 1.3-kb 3' *HindIII*-*EcoRI* fragment (44-1HS) of a 3.7-kb *mtp2* cDNA clone, 44-1, was used likewise. (The 44-1 clone has an anomalous 5' end containing repetitive sequence, and may not be full-length).

mtp1 BamHI digests



mtp2 EcoRI digests

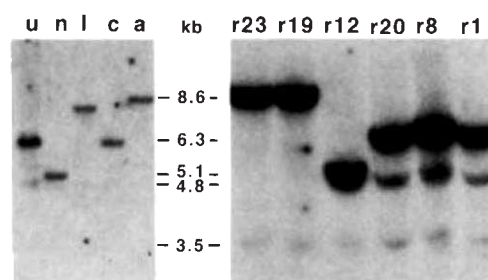


FIG. 2 Southern blots of genomic DNA from MHC congenic (a, c, l, n, u) and recombinant (r1, r8, r12, r19, r20 and r23) rat strains probed with the *mtp1* (510-15) (*a*) and *mtp2* (44-1HS) (*b*) cDNA probes described in the legend to Fig. 1.

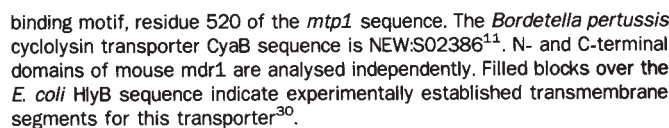
METHODS. All the rats used except r12 were congenic on the PVG background. The r12 haplotype is on a mixed background. Testis DNA (10 μg) from each rat strain was digested to completion with *BamHI* (*a*) or *EcoRI* (*b*), separated on 0.8% agarose gels and transferred to Duralon-UV (Stratagene) by capillary transfer. DNA was bound to the hybridization membrane by ultraviolet irradiation. Hybridization was performed at 65 °C in Church and Gilbert buffer³⁸, and washed for 60 min at 65 °C at a stringency of 1 × SSC in 1% SDS. In *b*, the nonpolymorphic 3.5-kb *EcoRI* fragment hybridizing weakly to the 44-1HS (*mtp2*) probe in the recombinant blot is part of the *mtp1* gene.

METHODS. The 510-15 insert was sequenced by nested deletions in both orientations in pBluescript KS(+) using the *ExoIII-ExoVII* method³⁹ and Sequenase (USB). The deletion in the 510-18 clone was identified by sequencing from a negative-strand primer located just downstream of the internal *Bam*H1 site at nucleotides 619-624.

A full-length cDNA of the *mtp2* gene has not so far been obtained, but sequence analysis of a fragment of 158 amino acids in the region immediately N-terminal to the nucleotide-binding domain has established that *mtp2* is related to *mtp1* (Fig. 4a).

The mtp proteins are attractive candidates for the hypothetical class I peptide transporter,⁵ as they are potentially capable of using both peptide translocating activity as shown by the yeast

structure relationships between the mtp1 polypeptide and other members of the ATP-dependent transmembrane transporter family. *a*, Dotplot comparisons between the amino-acid sequence of mtp1 (on the ordinate) and a series of transmembrane ABC transporters using the UWGCG programme COMPARE with a window of 30, a stringency of 16 and the default similarity table. The short matrix against mtp2 derives from sequence obtained from the 44-1 clone, yielding a 158-amino-acid open reading frame beginning about 1,500 nucleotides from the 3' end of the cDNA. Amino-acid similarities based on UWGCG GApped sequence comparisons for each ABC domain separately against mtp1 were as follows: mouse multidrug resistance protein mdr1 (sequence NEW:a25057²⁴) N-terminal domain residues 1-638, identity 29.5%, similarity 53.6%; C-terminal domain residues 639-1,276, identity 30.0%, similarity 54.1%; *Saccharomyces cerevisiae* α -type mating factor transporter STE6 (sequence GENBANK:X15428¹³) N-terminal domain residues 1-645, identity 23.2%, similarity 47.1%; C-terminal domain residues 646-1,290, identity 24.4%, similarity 47.9%; human cystic fibrosis conductance regulator CFTR (sequence NEW:A30300²²) N-terminal domain residues 1-740, identity 23.2%, similarity 47.1%; C-terminal domain residues 741-1,280, identity 22.4%, similarity 48.1%; *E. coli* haemolysin transporter HlyB (sequence PROTEIN:LEECB¹²) residues 1-707, identity 26.2%, similarity 50.1%; rat mtp2 residues 1-158, identity 31.0%, similarity 54.1%. *b*, Hydrophobicity plots of several transmembrane ABC transporters including mtp1 based on Eisenberg⁴⁰ values and plotted using the programme AMPHI (J. Coadwell, unpublished) with a window of 21 amino acids. The sequences are aligned on the G residue of the GKS nucleotide-



a-type mating factor transporter¹³, and the ability to accommodate structural variation in the transported compound, typical of the *mdr* proteins²³. If *mtp* proteins are indeed peptide transporters for class I, they would be expected to be localized in, and probably restricted to, the ER membrane. Certainly the unusual presence of a putative N-terminal signal sequence in *mtp1* is consistent with this localization, but a known C-terminal ER retention sequence for transmembrane proteins³² is missing. If *mtp* proteins are transporters for peptides of pathogenic origin they are likely to be under intense selection pressure, and would be expected, like class I MHC molecules themselves³³, to evolve an adaptive polymorphism. The localization of *mtp* genes to a notably polymorphic region of the MHC is consistent with such a property, as is the considerable divergence between mouse *HAM1* and rat *mtp1* sequences, especially towards the N terminus (Fig. 3). Finally, direct experiments are required to establish whether *mtp* genes and their mouse and human homologues indeed provide the genetic basis for the *cim* phenomenon and the properties of the RMA-S and CEM.174 cell lines, or whether other MHC-linked genes concerned with class I function remain to be discovered. □

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Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters

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CLASS I molecules of the major histocompatibility complex (MHC) bind and present peptides derived from the degradation of intracellular, often cytoplasmic, proteins, whereas class II molecules usually present proteins from the extracellular environ-

ment^{1–6}. It is not known how peptides derived from cytoplasmic proteins cross a membrane before presentation at the cell surface. But certain mutations in the MHC can prevent presentation of antigens with class I molecules^{7–9}. In addition, mutations possibly in the MHC can affect presentation by class II molecules¹⁰. Here we report the finding of a new gene in the MHC that might have a role in antigen presentation and which is related to the ABC (ATP-binding cassette) superfamily of transporters^{11,12}. This superfamily includes the human multidrug-resistance protein, and a series of transporters from bacteria and eukaryotic cells capable of transporting a range of substrates, including peptides.

Novel genes in the class II region of the human MHC have been isolated by preparing probes from genomic clones (either cosmids or YAC clones; J. Ragoussis and J. T., manuscript in preparation) from the MHC and probing these onto complementary DNA libraries. Attention was initially focused on probes from regions of genomic DNA containing clusters of restriction sites with CpG dinucleotides in their recognition sequence as

FIG. 1 Location of the *RING4* gene in the class II region of the human MHC. The upper panel shows the map of the human MHC class II region on the short arm of human chromosome 6 showing the main genes³⁴. The lower inset shows the position of the *RING4* gene between two *NotI* sites (N), separated by ~9 kb, in the class II region. This is the only area of the class II region that contains *NotI* sites. The region is covered by previously isolated cosmids U15 and U10 as well as YAC clones (ref. 35, J. Ragoussis *et al.* manuscript in preparation). Arrows indicate the direction of transcription and point towards the 3' end of the gene.

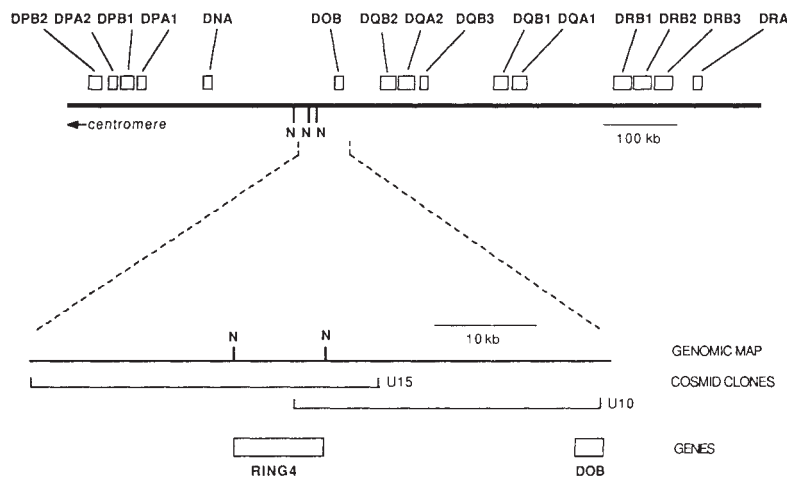
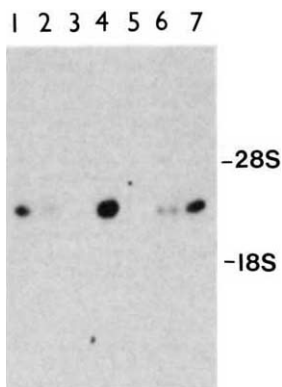


FIG. 2 Expression of *RING4* on northern blots. Total RNA was prepared from the following cell lines: 1, Raji (B lymphoblastoid cell line); 2, Molt 4 (T cell line); 3, SW1222 (colon carcinoma cell line); 4, SW1222 induced with γ -interferon; 5, SV80 (colon carcinoma cell line); 6, SV80 induced with γ -interferon; 7, DX3 (melanoma cell line). RNA was prepared and gels were run and blotted according to standard protocols³⁶. Interferon treatment was growth in 300 U γ -interferon per ml for 36–48 h before RNA extraction. A short cDNA clone (p1.2U) was used as a probe. This clone spanned the ~1,000 bp at the 3' end of p21U (Fig. 3). Autoradiography was carried out for 3 days using Kodak XAR-5 film.



these are often found near the 5' ends of genes¹³. So far, five new genes have been isolated, named *RING1* to *RING5* (I.H., A. Poustka and J.T., manuscript in preparation): here we present the sequence of *RING4*.

The position of this gene in the MHC is shown in Fig. 1. It lies at a CpG island which includes recognition sites for the enzyme *NotI*, between the HLA-DNA and -DOB genes, about

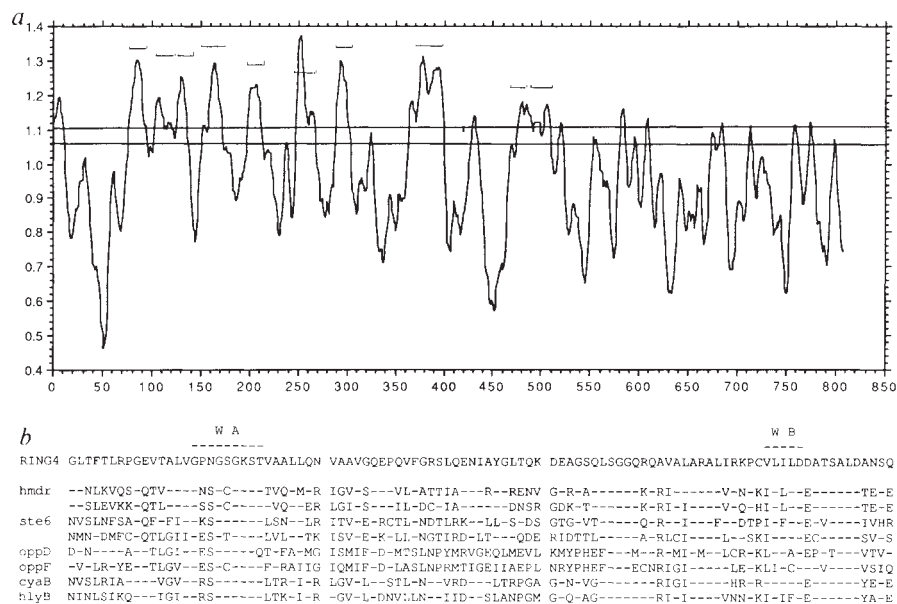
25 kilobases (kb) centromeric to the latter. Short probes were prepared from a 9-kb *NotI* fragment and used to screen cDNA libraries made from messenger RNA from a B lymphoblastoid cell line, JY, and a γ -interferon-stimulated monocyte cell line, U937. Several hybridizing cDNA clones were isolated and the longest sequenced. The amino-acid sequence revealed a long open reading frame, and sequencing of the *NotI* fragment used to derive the probes confirmed that it contained the *RING4* gene. Northern blots showed that an mRNA of appropriate length, about 2.8 kb, was expressed in B-cell lines and at a lower level in T-cell lines (Fig. 2). *RING4* mRNA was induced also in colon carcinoma cell lines treated with either γ - or α -interferon as well as in a melanoma cell line, DX3, that expresses products of the HLA-D region (Fig. 2 and J. John and G. Stark, personal communication). Primer extension analysis showed that the longest *RING4* cDNA was about 300 base pairs (bp) short of the 5' end of the mRNA (data not shown).

The sequence of the longest clone obtained from the U937 library is shown in Fig. 3. The derived amino-acid sequence revealed a protein that contains multiple blocks of hydrophobic amino-acids characteristic of transmembrane regions (Fig. 4a). Comparison of the *RING4* protein sequence with sequences in the EMBL database revealed that it belongs to the ABC superfamily of transporters^{11,12}, which includes the human and mouse multidrug-resistance proteins (MDR) (Fig. 4b). The protein

FIG. 3 Sequence of the *RING4* cDNA. The longest cDNA clone from the γ -interferon-induced U937 cDNA library, p21U, was sequenced using the dideoxy chain termination method³⁷. The cDNA library was in a derivative of the CDM8 vector and was prepared as described by Seed⁴¹. The open reading frame is shown encoding a protein of up to 808 amino acids (single-letter code). The first and second ATG (AUG) residues are arrowed. The first ATG is immediately preceded by an in-frame termination codon. Neither is particularly close to the consensus sequence of a eukaryotic translation initiation sequence (CC)A/GCCAUGG(G)³⁸. The vertical line is at the 5' end of the cDNA clone and the additional 5' sequence is obtained from overlapping genomic DNA, so it remains to be determined if the 5' end of the mRNA contains this sequence. The first AUG is followed by a potential hydrophobic signal sequence. Possible membrane spanning regions are overlined and a region that is highly homologous to the nucleotide-binding regions of related proteins is boxed (see Fig. 4b). Consensus N-glycosylation sites are marked by a dot. The square bracket at position 1,838 indicates three G residues. The p21U cDNA only contained two such residues at this position, causing an obvious deleterious frame shift. To confirm this, we sequenced three cDNAs from a B lymphoblastoid cell line, JY, and genomic DNA from the cosmid U15 (Fig. 1). All of these sequences contained three G residues and identical sequences flanking position 1,838. It has not been established whether U937 DNA has a similar frame shift in its *RING4* gene or whether it is the result of a cDNA cloning artefact. Multiple cDNA clones similar to p21U were obtained but one clone had an insert of several hundred base pairs at position 1,776 owing to differential splicing or failure of intron removal. The genomic organization will be published elsewhere (S.B. *et al.*, manuscript in preparation).

[illegible]

FIG. 4 a, Assignment of potential membrane-spanning regions in the derived *RING4* amino-acid sequence. The data were generated using a computer program written by M. Sternberg to use the algorithm of Rao and Argos to predict the location of membrane-spanning regions³⁹. The algorithm employs propensities for residues to be within a transmembrane region based on their observed frequency in such regions of several proteins. Regions favoured as membrane-traversing sequences are marked with horizontal bars and correspond to those indicated in Fig. 3. Note the potential short hydrophobic signal sequence. b, Comparison of the highly conserved regions of the ATP-binding folds of *RING4* and related gene products. Part of the region of *RING4* around the nucleotide-binding fragment is aligned with other related proteins from mammals and bacteria. The region shown is boxed in Fig. 3 with two gaps introduced. These alignments are not necessarily optimum for any two proteins from this set and other regions of homology were observed. Dashes indicate residues common to the *RING4* sequence. The Walker A and B nucleotide-binding motifs are shown above the sequence (refs 12, 14). Hmdr, human multidrug-resistance gene product³⁹; Ste6, yeast *STE6* gene product¹⁶; OppD and OppF were from *Salmonella typhimurium*¹⁵; HlyB is from



*E. coli*¹⁸; CyaB, *cyaB* gene product from *Bordetella pertussis*⁴⁰. Single-letter amino-acid code is used.

seems to consist of two distinct domains. The N-terminal domain is very hydrophobic and contains at least six possible membrane spanning α helices. The C-terminal domain is homologous to the ATP-binding cassette that is characteristic of this class of transporter^{12,14}. The *RING4* ATP-binding cassette is not significantly more closely related to those members of the family that transport proteins or peptides than to other members. But it will be particularly interesting to examine sequences in *RING4*, or other linked genes, for homology to the *OppA* gene which encodes part of the bacterial oligopeptide permease, because *OppA* encodes the peptide binding domain of the bacterial peptide transporter that binds to a wide range of peptide sequences before they are transported across the bacterial membrane.

Typically, eukaryotic ABC transporters consist of two hydrophobic domains and two ATP-binding domains¹¹. The *RING4* sequence encompassed only one of each unit. It therefore seems likely that *RING4* functions either as a homodimer, or as a heterodimer with a related protein. Preliminary experiments show that there are additional *RING4*-related sequences in the MHC. Compared with the other known ABC transporters, *RING4* has an extended N-terminal region, the total product consisting of 808 amino-acids, assuming the first AUG codon is used (Fig. 3). Also atypical is a potential signal peptide (Figs 3, 4).

The family of ABC transporters now includes over 30 members each specific for a different substrate¹¹. Substrates that are transported include sugars, inorganic ions, amino-acids, peptides and proteins. The oligopeptide permease in bacteria handles small peptides¹⁵; the *Saccharomyces cerevisiae* STE6 protein transports the a-factor pheromone^{16,17}; in *Escherichia coli* the *hlyB* gene product transports α -haemolysin, a protein of relative molecular mass ~107,000 (ref. 18), and related transporters export specifically other large polypeptides¹⁹. In none of these cases does the transported peptide molecule have a typical hydrophobic signal sequence.

The characteristic feature of the presentation of cytoplasmic antigens, such as influenza nucleoprotein, to class I-restricted T cells, is that the molecules presented lack hydrophobic signal sequences^{1,20}. Deletion of the N-terminal signal sequence of the influenza haemagglutinin can actually enhance presentation in certain conditions^{20,21}. A second feature is that rapid degradation of cytoplasmic antigens is associated with efficient presentation^{1,20,21}. This suggests that degradation to peptides may occur in the cytosol before their transport across the membrane of the

endoplasmic reticulum (ER). Consistent with this concept is the finding that short hydrophilic peptides expressed in the cytosol are presented efficiently to T cells²²⁻²⁴.

These features imply that a specialized peptide transport system may exist, possibly related to the ABC superfamily, that passes the peptide products of proteolysis from the cytosol into the ER^{1,25}. Recent evidence shows that association of peptides with the binding site on the class I heavy chain may be required for the assembly of stable class I molecules^{7,26-29}. The mutant cells RMA-S and 721.174 express unstable class I molecules, are unable to present cytoplasmic antigens, but do present extracellular peptides^{7,8,26,30-32}. This phenotype is consistent with loss of peptide transport from the cytosol into the ER^{7,26}, and in the case of 721.174 is associated with a deletion in the MHC between the DPA2 gene and the complement cluster³³. *RING4* is encoded within this deleted region and is therefore a candidate for at least one chain of a peptide transporter. □

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A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway

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MAJOR histocompatibility complex (MHC) class I molecules export peptides to the cell surface for surveillance by cytotoxic T lymphocytes¹⁻³. Intracellular peptide binding is critical for the proper assembly and transport of class I molecules⁴⁻⁶. This mechanism is impaired as a result of a non-functional peptide supply factor gene (*PSF*) in several human mutant cell lines with genomic lesions in the MHC. We have now identified *PSF* in the MHC class II region by deletion mapping in mutants and chromosome-walking. *PSF* is homologous to mammalian and bacterial

ATP-dependent transport proteins, suggesting that it operates in the intracellular transport of peptides.

The human B lymphoblastoid cell line (LCL) mutant 721.174 has been derived by γ irradiation and immunoselection against cell-surface class II molecules from LCL 721.45, in which a complete MHC haplotype of the parent LCL 721 had been already deleted^{7,8}. In mutant .174, most of the cytoplasmic class I heavy chains fail to associate with β_2 -microglobulin, resulting in a partial reduction of HLA-A2 and a complete loss of HLA-B5 at the cell surface^{9,10}. Exposure of .174 cells to peptides restores expression of HLA-A2 and -B5 on the cell surface, suggesting loss of function of an unknown peptide supply mechanism^{11,12}. Presumably, this is due to deletion of a gene(s), which is encoded in the MHC and is designated as peptide supply factor gene (*PSF*). From the single MHC haplotype retained in .45, the entire class II region except for the most centromeric *DPA2* and *DPB2* genes is deleted in .174 (ref. 13). This large homozygous deletion extends into the central MHC class III region between *DRA* and the steroid 21-hydroxylase B locus (*21-OHB*)¹⁴.

Initially, candidates for *PSF* were sought between *DRA* and *21-OHB*. To map the telomeric breakpoint of the .174 deletion, we isolated 200 kilobases (kb) of contiguous DNA in overlapping cosmids by chromosome-walking from *21-OHB* towards *DRA* (Fig. 1). A single-copy DNA fragment 60 kb downstream of *21-OHB* (probe a in Fig. 1) was present in .174, but a fragment 180 kb downstream of *21-OHB* (probe b in Fig. 1) was absent (data not shown). These two fragments therefore bounded a 120-kb segment containing the telomeric breakpoint of the .174 deletion. In this region, the frequent occurrence of *Bss*III and *Sac*II restriction sites suggested the presence of genes^{15,16}. By sequentially screening an HPB-ALL T-cell library with labelled

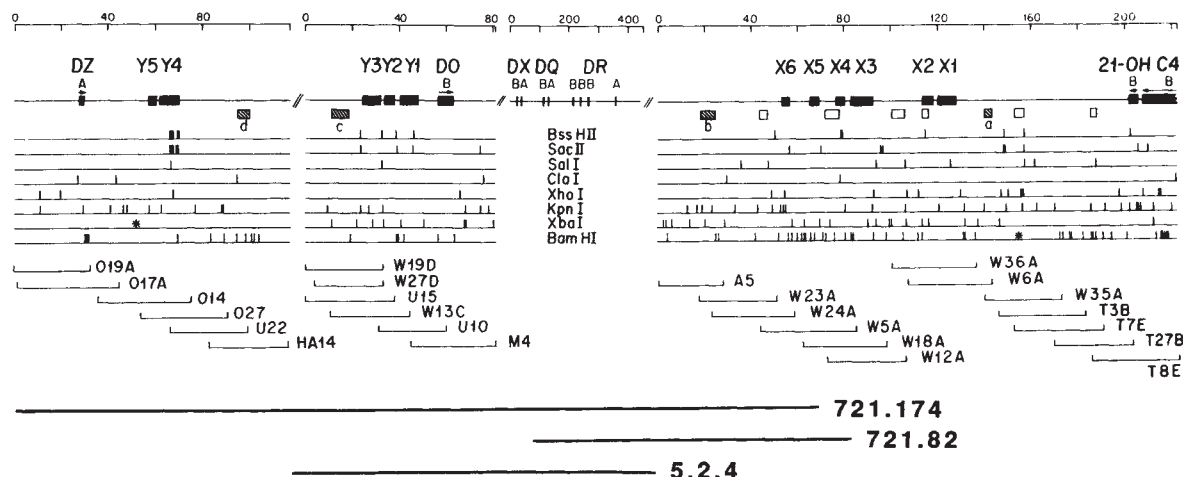
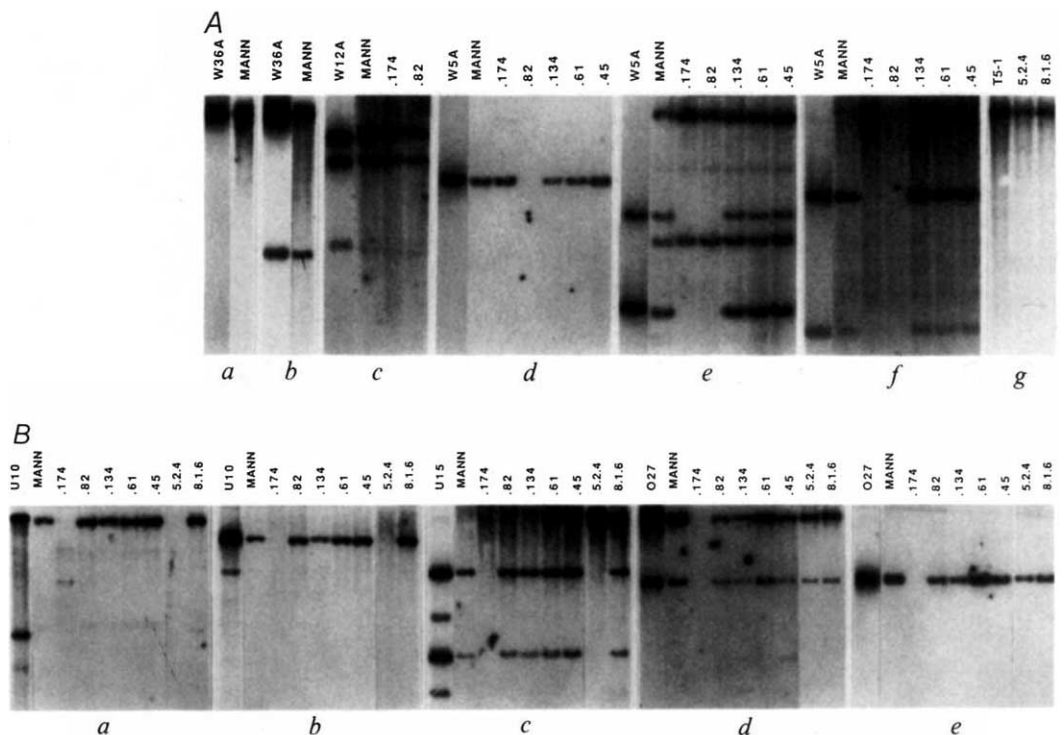


FIG. 1. Molecular structure of portions of the MHC class II and class III regions cloned in three series of overlapping cosmids. Top line, scale in kb. Bottom lines, homozygous deletions in the .174, .82 and 5.2.4 mutant LCLs. The cosmids from the class II region have been described previously^{17,18}. The cosmids from the class III region have been isolated in this study. Closed boxes, genes. Arrows show direction of transcription of genes. X1-X6 and Y1-Y5 were identified by isolation of cDNA clones with cosmid probes. The organization of the class II *DRA/B*, *DQA/B*, *DXA/B*, *DOB* and *DZA* genes and of *C4* and *21-OHB* in the class III region was established previously^{20,25}. Hatched boxes, genomic probes used to map deletion breakpoints in mutant

LCLs. Open boxes, probes used for chromosome-walking.

METHODS. The cosmid library from LCL MANN and the procedures involved in chromosome-walking are described in detail elsewhere^{26,27}. The first library screening was carried out with a C4 cDNA probe²⁵. A preannealing protocol was used to isolate X1-X6 and Y1-Y5 cDNA clones from a HPB-ALL T-cell library (a gift of B. Seed, Massachusetts General Hospital, Boston)²⁸ with total labelled cosmid inserts as probes²⁶. Sets of cDNA clones were matched by restriction mapping and DNA blot hybridizations. Selected cDNA probes were mapped to their corresponding genomic locations by hybridization to blots of cosmid DNA restriction digests.

FIG. 2 A, Localization of *X1-X6* in cloned cosmids (left lanes in *a-f*) and mapping of deletions in mutant LCLs by DNA blot hybridization. In *a-f*, all of the *X1-X6* cDNA probes hybridized to fragments in cosmids that were matched by bands in DNA from LCL MANN, of which the cosmid library had been constructed. In *e*, the *X5* probe detected two additional bands in total genomic DNAs, which corresponded to a homologous gene in a different chromosomal location. The .174 deletion breakpoint was between *X4* (*d*) and *X5* (*e*). The .82 deletion breakpoint was between *X3* (*c*) and *X4* (*d*). *g*, In 5.2.4, the cosmid probe *b* (Fig. 1) was not deleted. Restriction digests were with *Xba*I in *a* and *b* and with *Bam*HI in *c-g*. B, Localization of *Y1-Y5* in cloned cosmids (left lanes in *a-e*) and mapping of deletions in mutant LCLs by DNA blot hybridization. In *a-e*, all of the *Y1-Y5* fragments in cosmids were matched by bands in total genomic LCL MANN DNA. All of these were deleted in .174 and were present in .82. In 5.2.4, *Y1* (*a*), *Y2* (*b*) and *Y3* (*c*) were



deleted, but *Y4* (*d*) and *Y5* (*e*) were present. Restriction-enzyme digests were performed with *Bam*HI in *a*, *Xba*I in *b*, and *Kpn*I in *c-e*.

total cosmid inserts, numerous complementary DNA clones were isolated and mapped to their corresponding genomic locations. These locations in cosmids were matched by restriction fragments detected by blot hybridization in total genomic DNA (Fig. 2Aa-f). Thus, a cluster of six genes, *X1-X6*, was identified 70 kb centromeric of *21-OHB* (Fig. 1). *X1-X4* were present in .174 cells, but *X5* and *X6* were absent (Fig. 2Ac-f; note that in Fig. 2Ae, the *X5* cDNA detected two additional fragments in total genomic DNA that corresponded to another gene in a different chromosomal location). Thus, the telomeric breakpoint of the ~1,000-kb .174 deletion was between *X4* and *X5*.

Although *X5* and *X6* were formally candidate genes for *PSF*, we attempted to refine the localization of *PSF* by mapping deletions in additional mutant cell lines. LCL 721.82 shows normal HLA-A2 and -B5 surface expression and, therefore, is not deficient for *PSF*. As in .174, a large homozygous deletion in .82 includes portions of both the MHC class II and class III regions^{8,13,14}. The telomeric breakpoint of the .82 deletion was mapped between *X3* and *X4* (Fig. 2Ac, d). This ruled out *PSF* being located between *X4* and *DRA* in the class III region, as *PSF* was outside the .82 deletion. The centromeric breakpoint of the .82 deletion is between *DQB* and *DXA*¹³. Thus, a 300-kb segment from the class II region containing the telomeric *DRA/B* and *DQA/B* genes is deleted also in .82 (Fig. 1). *PSF* is therefore encoded between *DPA1* and *DQB*.

About 350 kb of the *DPA1-DQB* interval have been cloned in three series of cloned overlapping cosmids^{17,18}. These cosmids were examined for the presence of genes by screening the HPB-ALL library for homologous cDNA clones with selected total cosmid insert probes. Three genes, *Y1*, *Y2* and *Y3*, were identified 10 kb upstream of *DOB*. Two additional genes, *Y4* and *Y5*, were mapped 28 kb downstream of *DZA* (Fig. 1 and Fig. 2Ba-e). The relative orientation of the unlinked *DOB* and *DZA* genes was based on the alignment of the positions of two clusters of *Bss*HII and *Sac*II sites in the cosmids and in PFGE maps of this MHC region^{19,20}.

Predictably, *Y1-Y5* were deleted in .174 and present in .82 (Fig. 2Ba-e). An additional deletion mutant cell line, LCL 5.2.4,

was instrumental in the further selection of *PSF* candidates among *Y1-Y5*. This mutant has been isolated independently of .174 and .82 by chemical mutagenesis of the hemizygote LCL 8.1.6 and immunoselection against DR surface expression (E.M. and D.P., manuscript submitted). LCL 8.1.6 has been derived from the wild-type LCL T5-1 and lacks the *DRA/B*, *DQA/B*, *DXA/B* and *DOB* genes in one haplotype. In LCL 5.2.4, all of these genes are deleted also in the second haplotype. By all essential criteria—defective surface expression of the endogenous HLA-B27 heavy chains and recovery of normal expression by peptide-feeding—5.2.4 cells show the same phenotype as .174 cells, indicating that both lines lack *PSF* function (E.M. and D.P., manuscript submitted). The telomeric breakpoint of the 5.2.4 homozygous deletion was between *DRA* and the genomic probe *b*, which was located near the centromeric end of the cloned class III region cosmids (Fig. 1 and Fig. 2Ag). In the class II region, the centromeric breakpoint of the 5.2.4 homozygous deletion was between *Y3* and *Y4*. *Y4* and *Y5* were

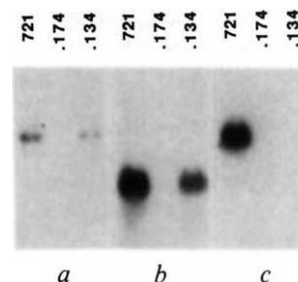


FIG. 3 Blot hybridization of RNA samples from LCLs 721, .174 and .134 with *Y1* (*a*), *Y2* (*b*) and *Y3* (*c*) cDNA probes. The *Y1*, *Y2* and *Y3* mRNAs were of 2.7, 1.4 and 2.7 kb, respectively. Hybridization was not detectable in .174, in which all of these genes are deleted. In .134, *Y1* (*a*) and *Y2* (*b*) mRNA was present, but *Y3* (*c*) mRNA was absent. METHODS. Total cell RNA preparations were obtained by the guanidinium thiocyanate method and centrifugation in cushions of 5.7 M cesium chloride²⁷. RNA samples (15 µg) were fractionated by electrophoresis in agarose-formaldehyde gels before blot transfer.

a 1: GGGTCGGGATAGCACCAGGCTACTGCACTGGGGAAGTCAACCTACCGCTCGTGTG
 1: G S A D S T R L L H W G S H P T A F V V
 61: AGTTATGCAGCGGCACTGCCGAGCAGCCCTGGGCACAACTCGGAGCCTCGGTG
 21: S Y A A A L P A A A L V H K L G S L W V
 121: CCGGCGGTGAGGCGGCTCTGGAAACCTGTGGTGGCTTCTAGGCTCCTGGGCTG
 41: P G G Q G G S G N P V R R L L G C L G S
 181: GAGACGCGGCTCTGGCTGTCTCTGGTCTGGTCTCTCTCTCTGGGAGATG
 61: E T R R L S L F L V L V L S S L G E M
 241: GCCATTCATCTTACGGGCGGCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 81: A I P F F T G R L T D V I L Q D G S A D
 301: ACCTTCACGAACTTAACTCTCATGCTCACTTCCACCATGCCAGTGCAGTCTGGAG
 101: T F T R N L T L M S I L T I A S A V L E
 361: TTCGTTGGTGACGGATCTATAACAACCATGGGCCAGTGCACAGCCATTCAGGGA
 121: F V G D G I Y N N T M G H V H S H L Q G
 421: GAGGTGTTGGGCTGCTCTGGCGAGGACGAGTTCCTCAACAGAACGACAGGT
 141: E V F G A V L R Q E T E F F Q Q N Q T G
 481: AACATCATGCTCGGTAACAGAGGACACCTCCACCTGAGTATTCTCTGAGTGAAT
 161: N I M S R V T E D T S T L S D S L S E N
 541: CTGAGCTATTCTGTGTACCTGGTGGGAGGCTATGCTCTTGGGATCATGCTCTGG
 181: L S L P L W Y L V R G L C L L G I M L W
 601: GGATCAGTCTCCCTCACCATGTCACCTGATCACCCTGCTCTGCTTCTCTGCCC
 201: G S V S L T M V T L I T L P L L F L L P
 661: AAGAAGTGGGAAATGGTACCACTGCTGGAAGTGCAGTGGGGAATCTCTGGCAA
 221: K K V G K W Y Q L L E V Q V R E S L A K
 721: TCCAGCAGTGGCATTGAGGCTCTGCGGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 241: S S Q V A I E A L S A M P T V R S F A N
 781: GAGGAGGCGGAGCCAGAACTTGGGAAAGTGCAGAAATAAGACACTCAACAG
 261: E E G E A Q K P R E K L Q E I K T L N Q
 841: AAGGAGGCTGGCTATGACGACCTCTGAGGACCTAGTATTTCAGTATGCTGCTG
 281: K E A V A Y A V N S W T T S I S G H L L
 901: AAAGTGGGAATCTCTACATGCTGGGAGCTGGTACCAGTGGGCTGTAAGCAGTGG
 301: K V G I L Y I E A L S A M P T V R S F A N
 961: AACCTGTACATTTGCTCTACAGAGTCACTGCTGAGGCTGCTGAGGCTGCTGCTG
 321: N L V T F V L Y Q M F T Q A V E V L L
 1021: TCCATCTACCCAGAGTACAGAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 341: S I Y P R V Q K A V G S S E K I F E Y L
 1081: GACCGCAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 361: D R T P R C P P S G L L T P L H L E G L
 1141: GTCCAGTCCAAGATGCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 381: V Q F Q D V S F A Y P N R P D V L V L Q
 1201: GGGCTGACATTCACCTACGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 401: G L T P T L R P G E V T A L V G P N G S
 1261: GGAAGAGCAGTGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 421: G K S T V A A L L Q

b
 PSF(Y3) : LTPHLEGLVQFDVSFAFYNRPDLVLQGLTFTLRPEVTALVQNGSKSTVAALL
 MDR : -M-NT--N-T-GE-V-N-T--IP-----SLEVKK-OTL--SS-C--VQ--
 MDR3 : -K-DKF--NIT-NE-V-N-T-A-N-P-----SLEVKK-OTL--SS-C--VQ--
 OppD : -TAVND-N-----A--TLGI--ES--
 HisP : -K-VSLQA-A-D-ISII--SS--
 MalK : -SKDINLDIHD--FVVF--S-C--
 RbsA : -G-KA-S-AALNVY--R-M--E-A--MHK--
 CFTR : -ENIS-SIS--QRVG-LRT--

c
 PSF(Y3) : FGAVLRQETFEFFQONOTGNIMSRVTEDTSTLSLSLSENLFLWYLVRLCLLGLMLWGS
 MDR : -H-IM--IGV-DVHDV-ELNT-L-D-V-KINEVIGKIGM-FOSMAFTFTGFIQVGTGR
 PSF(Y3) : VSLTHTVTLITLPLFLPLPKVKYQVLLVQVRESLAKSSQVAIEALSAMPTVRSFANE
 MDR : WK--L-I-AIS-V-G-SAAVVA-ILSFTDKELLAY--AGA--E-V-A-IR--IA-GGOK
 PSF(Y3) : GEAQKREKLEIKTLNOKEAVA
 MDR : K-LERYNKN-E-A-RIGI-K-IT

FIG. 4 **a**, Sequence of a partial 1,290-bp Y3 cDNA. Sequence was obtained by the dideoxynucleotide chain termination method from both strands of restriction fragments subcloned into M13. Amino acids below nucleotides are denoted by the single-letter code. **b**, Homology of PSF (Y3) to mammalian and bacterial transport proteins around an ATP-binding consensus, GXXXXGKS (boxed)²¹⁻²⁴. Dashes, identical residues. The computer homology search of the National Biomedical Research Foundation Protein Sequence Data Base employed an alignment program by Fing and Doolittle²⁹. **c**, Homology to MDR. There is, 142 residues from the beginning of the partial Y3 sequence, a stretch of 142 amino acids showing 25% homology to the sequence of MDR (residues 152-294) corresponding to two transmembrane domains. Dots indicate similar charge of amino acids. Single-letter amino-acid code used.

present in 5.2.4, but Y1, Y2 and Y3 were deleted (Fig. 1 and Fig. 2Ba-e). Thus, all of the deletion-mapping data obtained from the different mutants are in agreement and indicate that PSF function is encoded by Y1, Y2 or Y3.

To identify PSF among Y1-Y3, two additional mutants were investigated for genomic lesions. LCL 721.61 and LCL 721.134 also originated from the one haplotype-less mutant .45 and have been selected for absence of surface class I molecules^{7,8}. Although .61 and .134 cells have not been characterized as well as .174 cells, lack of PSF function is shared by all of these mutants⁹. In contrast to .174 and 5.2.4, homozygous deletions have not previously been demonstrated in .61 and .134. Indeed, using all of the cDNA probes for X1-X6 and Y1-Y5, we failed to detect any deletions in these mutants (Fig. 2Ad-f and Ba-e). However, RNA blot hybridization showed that Y3 messenger RNA was absent from .134 cells, although the closely linked Y1 and Y2 genes were transcribed at normal levels (Fig. 3a-c). This defect identified Y3 as PSF with near certainty. This gene may have been inactivated in .134 by a small deletion or point mutation in a transcriptional control element.

The Y3 mRNA was of about 2.7 kb (Fig. 3c). A partial cDNA sequence of 1,290 base pairs (bp) truncated at both ends was translated and analysed by a homology search of the National Biomedical Research Foundation Protein Database (Fig. 4a,b). A segment of 58 amino acids at the end of the sequence showed

different degrees of homology to several proteins that function in active transport. These include mammalian multidrug-resistance proteins (MDR)^{21,22}, the cystic fibrosis transmembrane conductance regulator (CFTR)²³, the bacterial oligopeptide permease membrane protein (OppD) and other components of bacterial periplasmic transport systems (HisP, MalK and RbsA)²⁴. All of these proteins share an ATP-binding consensus sequence, which is conserved also in Y3. Also, 142 residues from the beginning of the partial Y3 sequence, there is a stretch of 142 amino acids that has 25% homology to a sequence in the human MDR protein (residues 152-294) that corresponds to two transmembrane domains (Fig. 4c)²¹. Moreover, on the basis of the overall similarity between hydrophobicity profiles of the available Y3 and MDR amino-acid sequences, Y3 may contain as many as six transmembrane segments. Y3 may therefore be a membrane protein with several membrane-spanning domains and function in energy-dependent transport. This supports the identification of Y3 as PSF which may thus operate in the translocation of peptides from the cytosol into the endoplasmic reticulum, as proposed originally by Townsend *et al.*⁵. PSF may have evolved specifically to provide class I molecules with peptides. This would suggest that PSF is selective for peptide length and/or sequence, although its role may be more general. The location of PSF in the MHC may exemplify an evolutionary strategy for the co-selection of functionally related genes. □

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Germ-line transmission of a mutated *p53* gene in a cancer-prone family with Li-Fraumeni syndrome

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TUMOUR suppressor genes, whose usual function seems to be controlling normal cell proliferation^{1,2}, have been implicated in many inherited and sporadic forms of malignancies (for reviews, see refs 3 and 4). Much evidence supports the concept of tumour formation by loss-of-function mutations in suppressor genes, as predicted by the two-hit model of Knudson⁵ and DeMars⁶. The suppressor gene, *p53*, is affected in such a manner by numerous mutations, which occur in a variety of human tumours^{7–10}. These mutations usually represent the loss of one allele and the substitution of a single base in the other. We have now analysed the *p53* gene in a family affected by Li-Fraumeni syndrome¹¹, a rare autosomal dominant syndrome characterized by the occurrence of diverse mesenchymal and epithelial neoplasms at multiple sites. In some instances the neoplasms seem to be related to exposure to carcinogens, including ionizing radiation. The Li-Fraumeni family that we studied had noncancerous skin fibroblasts (NSF) with an unusual radiation-resistant phenotype^{12–15}. DNA derived from the NSF cells of four family members, spanning two generations, had the same point mutation in codon 245 (GGC→GAC) of the *p53* gene. This mutation leads to substitution of aspartic acid for glycine in one of the regions identified⁹ as a frequent target of point mutations in *p53*. The NSF cell lines with the mutation also retained the normal *p53* allele. This inherited *p53*

mutation may predispose the members of this family to increased susceptibility to cancer.

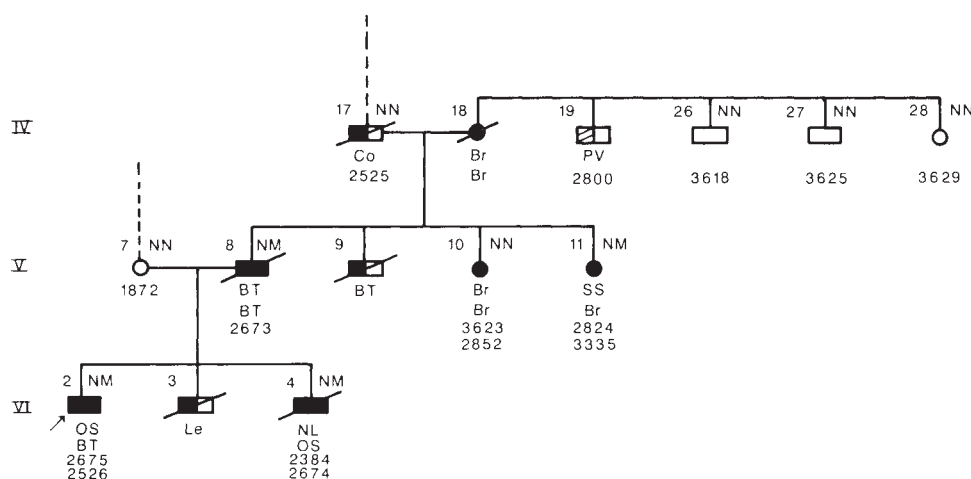
Eighteen individuals from six generations of the Li-Fraumeni family that we studied had malignancies. No karyotypic abnormalities in the family members have been previously identified. The diversity of malignancies in the specific cancer-prone branch of the family that we studied (see Fig. 1 for a partial pedigree) suggested a defect in the regulation of growth of epithelial and mesenchymal cells¹⁴. Although *p53* mutations have previously been identified only in tumour tissues, we reasoned that if a mutation in the *p53* gene were central to the development of neoplasia in this family, the mutation might be present in non-cancerous somatic cells. We used NSF cell lines derived from members of three generations of the family to assess mutations in selected regions of *p53*.

Four regions in *p53* have been identified as the most frequent targets of point mutations⁹: region A, encompassing codons 132–143; B, codons 174–179; C, codons 236–248; D, codons 272–281. All four regions are located in the areas of the gene encoding the most highly conserved parts of the protein. Using the polymerase chain reaction (PCR)⁹, these regions were amplified from DNA of family members' NSF lines. The first PCR reaction yielded a 2.9-kilobase (kb) DNA fragment which contained all four mutational 'hot-spot' regions. This fragment was then reamplified using a set of primers encompassing regions A and B and a second set of primers encompassing regions C and D. The nucleotide sequences of these regions were then determined, leading to the identification of a single base substitution, a G→A transition, in codon 245 of *p53* (Figs 2 and 3). This mutation, which results in the substitution of an aspartic acid for glycine in the *p53* protein, was found in DNA from four different NSF cell lines of the family: 2675, 2674, 2673 and 3335. These cell lines were obtained from the proband (VI-2), his brother (VI-4), their father (V-8) and a paternal aunt (V-11), respectively. Individuals were heterozygous for this mutation, one allele retaining the normal GGC sequence. It is important to note that all four of these individuals had cancer (Fig. 1). This mutation was not observed in the NSF DNAs from a second paternal aunt (2852, V-10) with breast cancer or from a genetically unrelated normal control. More importantly, the mutation was not found in NSF DNAs derived from the mother (1872, V-7), or the paternal grandfather (2525, IV-17) of the proband,

TABLE 1 *p53* in a cancer-prone family

Fibroblast cell line	Individual	Relationship to proband	Sequence
196		Unrelated normal control	Codon 245 GGC
2675/2526	VI-2	Proband	Codon 245 GGC→GAC (Gly→Asp)
2674/2384	VI-4	Brother	Codon 245 GGC→GAC (Gly→Asp)
2673	V-8	Father	Codon 245 GGC→GAC (Gly→Asp)
3623/2852	V-10	Paternal aunt	Codon 245 GGC
2824/3335	V-11	Paternal aunt	Codon 245 GGC→GAC (Gly→Asp)
1872	V-7	Mother/normal spouse	Codon 245 GGC
2525	IV-17	Paternal grandfather/spouse	Codon 245 GGC
3618	IV-26	Paternal great uncle	Codon 245 GGC
3625	IV-27	Paternal great uncle	Codon 245 GGC
3629	IV-28	Paternal great aunt	Codon 245 GGC

FIG. 1 Partial pedigree of a cancer-prone family. Shown here is a branch of a much larger pedigree in which cancer can be traced through six generations in three separate lineages from a woman who died with breast cancer in 1865 (ref. 12). Normal skin fibroblast (NSF) cell line designations are given for each individual, where available. NM, individuals in whom the G→A transition in codon 245 of one *p53* allele was found; NN, presence of two normal alleles; ■ (arrowed), double cancer in proband; ●, single primary cancer in deceased female. Abbreviations: OS, osteogenic sarcoma; SS, soft-tissue sarcoma; BT, brain tumour; Br, breast cancer; PV, polycythaemia vera; Le, leukaemia; Co, colon cancer; NL, neurilemmoma.



both of whom married into the cancer-prone lineage. Unfortunately, neither NSF cell lines nor lymphocytes from the deceased paternal grandmother of the proband in the cancer-prone lineage, who died from bilateral breast cancer, were available for analysis. DNAs from NSF cell lines of two paternal great uncles and a great aunt (Fig. 1), all unaffected, did not reveal a mutation in codon 245 (Table 1). Moreover, a separate isolate of NSF cells from the proband, his brother and their aunt possessed the same mutation as that detected in the original cell lines, confirming that the mutation was genetically inherited rather than an artefact of cell culture. The finding that the mother (V-7) and paternal grandfather (IV-17) of the proband did not possess the mutation lends credence to the conclusion that the proband and his brother acquired this mutation genetically from their father. In addition, the father and his sister (aunt V-11) most probably inherited the mutation from their mother (IV-18) who is directly in the lineage of the cancer-prone family. Furthermore, that an antibody specific for mutant *p53* protein¹⁶ recognizes the *p53* protein in a NSF cell line of the proband (S.S., V. Sykes, D. Krishnakumar, K.F.P. and E.H.C., unpublished results), strongly suggests that the mutation has a functional effect. The finding of a heritable mutation in the *p53* gene is analogous to similar mutations in somatic cells of individuals with a defective *Rb* gene and predisposition to retinoblastoma (for a review, see ref. 3), suggesting that a heritable defect in the *p53* gene in the family that we studied results in a heightened risk of cancer. Two independent NSF cell lines from one of the paternal aunts with breast cancer, V-10, did not have the *p53* mutation in codon 245. With mendelian inheritance of a specific genetic trait, one would not expect it to be carried by every individual in a generation. Although bilateral breast cancer has been considered to be one of the primary characteristics of the Li-Fraumeni Syndrome, the previously identified elevated *c-myc* expression in the NSFs of this family¹⁴, as well as the contribu-

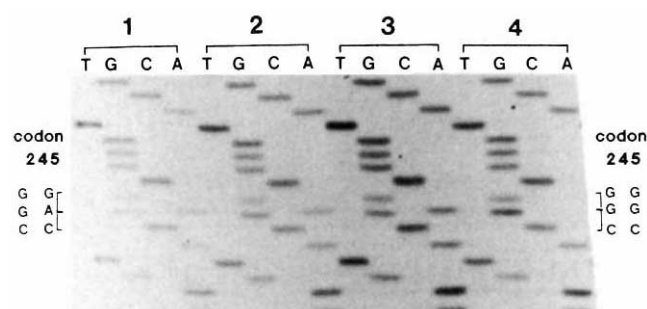


FIG. 2 Identification of a point mutation in the *p53* gene of NSF cell lines from members of a cancer-prone family. Shown is a representative sample of the sequence data obtained from seven different NSF cell lines representing four individuals in the cancer-prone family, as described in the text. The sequence data shown is for the area surrounding and including codon 245 where the point mutation was observed and is identical for all seven cell lines. The region of the *p53* gene that includes all four mutational hot spots was amplified by PCR using high relative molecular mass DNA derived from a fibroblast cell line of the proband, 2675 (1), his brother, 2674 (2), their father, 2673 (3) and a genetically unrelated normal control, 196 (4). Using PCR primers P3 and P4, as described by Nigro *et al.*⁹, a 2.9-kb primary PCR product was obtained. Using PCR primers 5'-TTCCTCTCCTGCACTACTCC-3' (sense), and 5'-CGG-AATTCAGCGGCTCATAGGC-3' (antisense), a region including hot-spots A and B was amplified by asymmetric PCR. Similarly, a region covering hot-spots C and D was amplified by PCR primers 5'-CAGGTCTCCCCAAGCGCACTGGCC-3' (sense), and 5'-CATCGAATTCTGGAACTTTCCACTTGAT-3' (antisense). The single-stranded DNA generated by asymmetric PCR was purified and the regions of interest sequenced by complementary sequencing primers using a Sequenase kit (USB). Each sample reported was sequenced at least twice from sequencing templates generated from two different high relative molecular mass DNA preparations. The sequence of codon 245 for each allele of the family NSF cell lines is shown on the left, whereas the sequence of codon 245 for each allele of the normal control is shown to the right of the sequence.

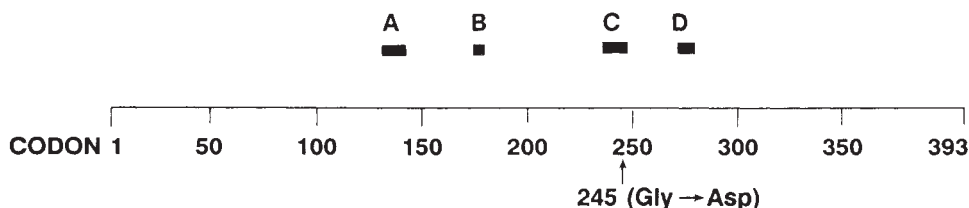


FIG. 3 An inherited *p53* point mutation in a cancer-prone family. The arrow indicates the location of the point mutation found in four members of the Li-Fraumeni family under study. Regions A, B, C and D (codons 132-143,

174-179, 236-248 and 272-281, respectively) where point mutations in human *p53* are clustered are indicated by the black squares. (Based on Nigro *et al.*⁹.)

tion of additional genetic influences inherited from her father's (IV-17) branch of the family, might contribute to her susceptibility to bilateral breast cancer. In fact, other incidences of cancer have been reported in the pedigree of IV-17 (ref. 12). More extensive sequencing of the *p53* gene, encompassing areas beyond the four hot-spot regions, should provide further clarification.

It has been suggested that point mutations in the conserved region of the *p53* gene can cause conformational changes in the encoded nuclear phosphoprotein. Mutant *p53* protein forms a complex with wild-type *p53*, thereby inhibiting the normal functions of the latter^{17,18}. Furthermore, mutations in wild-type *p53* protein can abolish the transformation-suppressing activity of the normal *p53* in a *ras* complementation assay¹⁹. The addition of wild-type *p53* complementary DNA can suppress the growth of human glioblastoma²⁰ and colorectal carcinoma²¹ cells. Although the precise functions of the *p53* protein remain to be elucidated, several studies of the interaction between *p53* protein and viral oncoproteins such as the simian virus 40 T antigen^{22,23}, adenovirus E1b²⁴, and papilloma virus E6²⁵, or studies of the functions of *p53* involving the microinjection of anti-*p53* antibody²⁶, indicate that *p53* may be essential for the control of cell proliferation. Because affected individuals of the family we studied often have more than one type of neoplasm, a defect in a gene such as *p53*, which is expressed in a variety of cell types, might disadvantage cells of these individuals in terms of the control of normal cell proliferation. Additionally, this mutation in *p53* was found only in NSFs of affected individuals.

Comparisons between normal and tumour tissue from patients with non-hereditary colorectal⁷ or lung cancer⁸, have demonstrated that neither allelic loss nor *p53* mutations occur in these patients' unaffected cells. The inherited nature of the *p53* mutation that we have now identified in noncancerous cells may represent an early genetic alteration having a predisposing effect in carcinogenesis in the family we studied.

Note added in proof: While this paper was being accepted for publication, similar findings in Li-Fraumeni syndrome were reported²⁷. □

Site-independent expression of the chicken β^A -globin gene in transgenic mice

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THE level of expression of exogenous genes carried by transgenic mice typically varies from mouse to mouse and can be quite low. This behaviour is attributed to the influence of the mouse chromatin near the site of transgene integration^{1,2}. This 'position effect' has been seen in transgenic mice carrying the human β -globin gene. It was however, abolished when DNase I hypersensitive sites (normally found 65 to 44 kilobases (kb) upstream) were linked to the human β -globin transgene³⁻⁵. Thus, the upstream DNA (previously named a dominant control or locus activation region, now denoted a locus control region) conferred the ability to express human β -globin at high levels dependent on copy number on every mouse carrying the construct. We report here an investigation of chicken β^A -globin gene expression in transgenic mice. A 4.5-kb fragment carrying the β^A -globin gene and its downstream enhancer⁶⁻⁹, without any far upstream elements, is sufficient to ensure that every transgenic mouse expresses chicken globin messenger RNA at levels proportional to the transgene copy number. Thus the chicken DNA elements that allow position-independent expression can function in mice. In marked contrast to the human β cluster, these elements are no farther than 2 kb from the gene. The location of the elements within the cluster demonstrates that position independence can be mediated by DNA that does not define a gene cluster boundary.

To examine the elements contributing to tissue-specific and integration site-independent expression, we produced transgenic mice containing the chicken β^A -globin gene with ($\beta^A E$) or without ($\beta^A \Delta E$) the downstream enhancer (Fig. 1a). Six of 33 mice developing from embryos injected with $\beta^A E$, and 5 of 62 mice developing from $\beta^A \Delta E$ -injected embryos carried the transgene. All transgenic mice carried intact β^A -globin genes, integrated in head-to-tail tandem arrays (Fig. 1b, and data not shown). Breeding resulted in the establishment of five $\beta^A E$ lines (one founder did not breed) and six $\beta^A \Delta E$ lines (one founder was not bred, and another contained three independently segregating integration sites).

Transgene expression was measured by primer extension. Correctly initiated β^A mRNA was detected in RNA isolated from blood of all six $\beta^A E$ lines, but not in non-transgenic mice, nor in any of the $\beta^A \Delta E$ mice (summarized in Table 1). β^A -globin RNA present at 0.02% of the mouse β^{maj} -globin RNA abundance would have been detected. Pretreatment of the mice with phenylhydrazine¹⁰ increased the total RNA yield 8- to 12-fold (to $\sim 1 \mu\text{g } \mu\text{l}^{-1}$ blood), but did not affect the relative expression of the mouse α , mouse β^{maj} , and chicken β^A genes. β^A -globin RNA expression was erythroid-specific (present at high levels in blood and spleen; Fig. 2a). We presume that the β^A RNA in non-erythroid tissues is due to blood contamination as the $\beta^A : \alpha : \beta^{maj}$ ratio was constant (Fig. 2a).

We were curious as to what developmental pattern β^A would exhibit in the mouse. In the chicken, the β^A gene is first expressed in yolk sac in mid-stage embryos and is the predominant β -globin thereafter¹¹. In both transgenic mouse lines tested (Fig. 2b), the β^A transgene is expressed in 11.5-day embryos (when all of the embryonically derived erythroid cells are produced in

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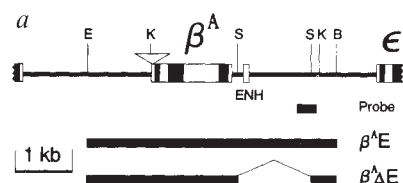
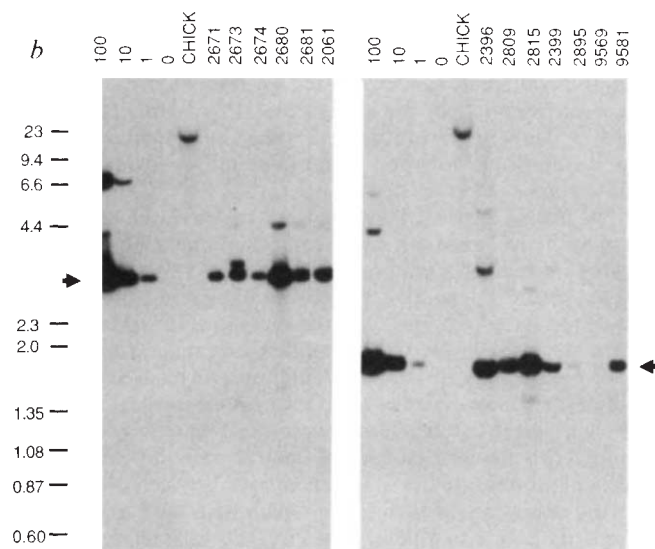


FIG. 1 Production of transgenic mice. *a*, Map of the chicken β -globin locus. The β^A and ϵ genes (transcribed from left to right), β^A/ϵ enhancer (ENH), DNA fragments ($\beta^A E$, $\beta^A \Delta E$) used for making transgenics, and hybridization probe are indicated. $\beta^A E$ is a 4.45-kb fragment extending from 1.05 kb upstream of the β^A transcription start site to 0.94 kb 5' of the ϵ start site. It contains a 35-base pair (bp) insert with a new *KpnI* site in the 5', transcribed, untranslated region of the β^A gene²⁶. $\beta^A \Delta E$ is the same as $\beta^A E$, except for a 1.25-kb *SacI* deletion that begins ~90 bp 3' of the polyadenylation site and removes the β^A/ϵ enhancer. The probe, I142, is a 362-bp *BglII/KpnI* fragment. Restriction sites are: E, *EcoRI*; K, *KpnI*; S, *SacI*; and B, *BamHI*. *b*, Southern blots of DNA from transgenic mice. Transgenic FVB/N mice were produced by injection of DNA²⁷, their tail DNA was digested with *KpnI*, and subjected to Southern blotting. Left panel, $\beta^A E$ founder animals (except for 2061 which is derived from 2702). Right panel, $\beta^A \Delta E$ founder animals and two 2396-derived progeny (2809, carrying integration site A, and 2815 carrying sites A and C). Arrows show the fragments expected from intact, unrearranged transgenes. Fragments smaller than the arrows are derived from rearranged copies of the transgenes. The 4.5-kb (left panel) and 3.2-kb (right panel) bands are the products expected from partial digestion of head-to-tail tandemly integrated transgenes. Non-transgenic mouse DNA augmented with plasmids containing $\beta^A E$ (left panel) or



$\beta^A \Delta E$ (right panel) at the indicated copy number (per diploid genome) is in lanes marked 0, 1, 10, and 100 (bands larger than those indicated by the arrows are partially digested plasmids). Chicken genomic DNA is also shown at a loading corresponding to a copy number of four; the hybridizing band is of the appropriate size as genomic DNA does not carry the inserted *KpnI* site. The positions of size markers, in kilobases, are at the left.

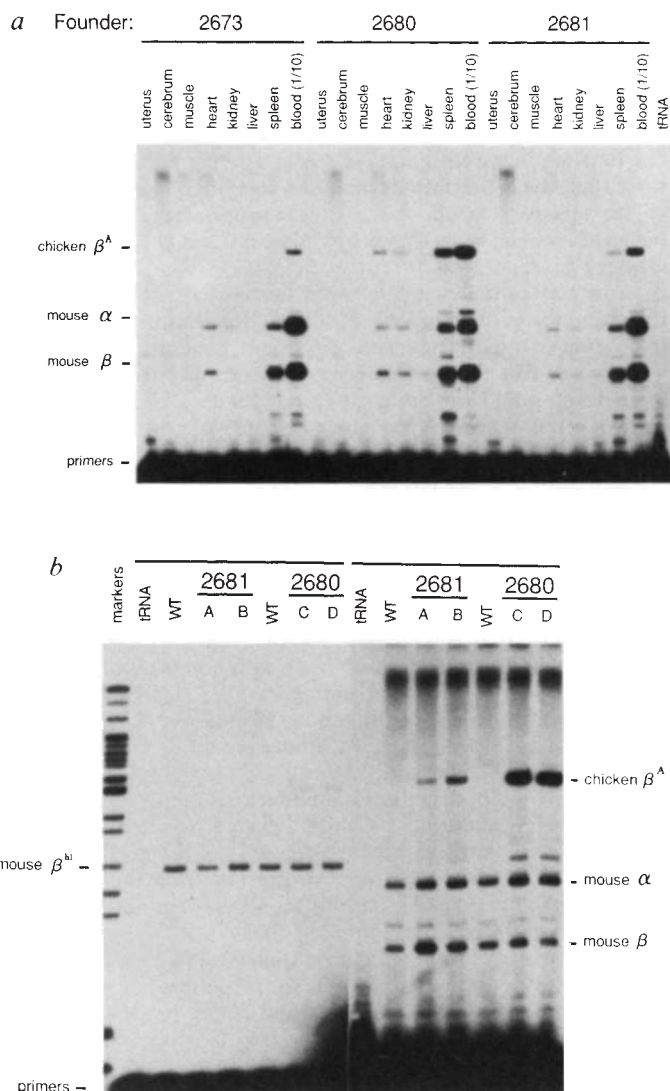


FIG. 2 Tissue and developmental specificity of transgene expression. *a*, RNA was isolated from the indicated tissues of adult female mice (derived from the indicated founders) and measured by primer extension. The chicken β^A , mouse α , and mouse β^{maj} products are so indicated. *b*, Primer extension using total RNA from 11.5-day-old embryos (including placenta and membranes; at 11.5 days all the embryonically produced blood is derived from the yolk sac¹²). The left lanes show primer extensions using the mouse embryonic β^{h1} primer with, from left to right: tRNA, a negative control template; three litter mates, one non-transgenic (WT) and two transgenic (A and B) from breeding a 2681-derived male with a non-transgenic female; and three litter mates, one non-transgenic (WT) and two transgenic (C and D) from breeding a 2680-derived male with a non-transgenic female. The right lanes use the chicken β^A , mouse α , and mouse β^{maj} primers with the same RNAs. Measurement of β^{h1} RNA allows estimation of the contribution of the embryonic erythroid tissue to the total RNA isolated. **METHODS.** RNA was isolated²⁸, primer extension¹³ performed with 10 μ g (1 μ g for blood) of RNA, and the products separated on denaturing 8% polyacrylamide gels. The primers and product sizes are: chicken β^A , 5'-GGTGATGAGCTGCTCTCTCTC-3', 115 nucleotides; mouse α , 5'-CAGGCAGCCTTGATGTTGCTT-3', 65 nucleotides; mouse β^{maj} , 5'-TGATGCTGTCTTCTGGGGT-TGTG-3', 53 nucleotides; and mouse β^{h1} , 5'-ATAGCTGCCTTCTCTCTCAGCT-3', 87 nucleotides. the primers are specific for the indicated mRNAs, except that the β^{h1} primer also detects β^A mRNA. The chicken primer detects the other chicken β -like globins, none of which is present in mice. Assays using a single primer gave identical results to those using multiple primers.

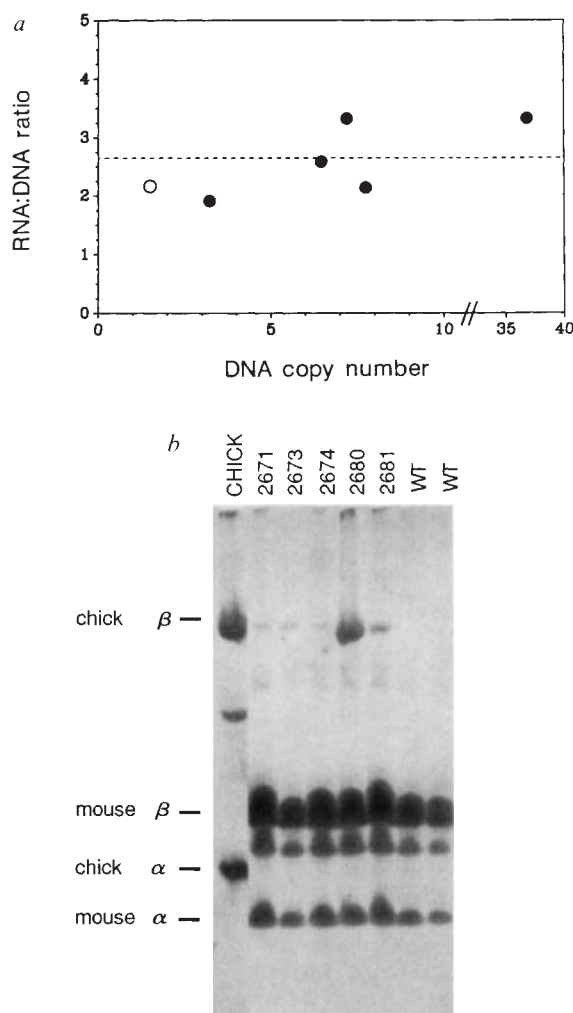


FIG. 3 Copy number dependence of RNA expression. *a*, β^A RNA abundance divided by DNA copy number plotted against DNA copy number. Solid circles represent the mean progeny data and the open circle is the datum for founder mouse 2674 (data from Table 1). The RNA:DNA ratio is the amount of transgene RNA, per transgene copy, expressed as a percentage of the amount of mouse β^{maj} RNA, per gene copy (assuming equally efficient priming and reverse transcription of the different primers and 2 β^{maj} genes per diploid genome). The β^A mRNA abundance per copy averages 2.7% that of mouse β^{maj} . *b*, Triton/acid/urea gel electrophoresis of erythrocyte lysates. A Coomassie-stained gel with lysates from five founder mice, two non-transgenic (WT) mice, and an adult chicken (CHICK) is shown. Positions of the various globins are as indicated. Lysate preparation and electrophoresis were essentially as described²⁹. The FVB/N strain used here carries the 'diffuse' (*Hbb^d*) allele (C. Hansen, personal communication).

the yolk sac)¹². Thus in the mouse, β^A expression begins in the yolk sac and continues through adulthood (Fig. 2*b* and data not shown).

To determine whether locus control region (LCR) activity was present in the β^A fragment, we measured the transgene DNA copy number and RNA abundance (Table 1). Mouse α -globin mRNA served as an internal control for measurement of β^A RNA, confirming equal amounts of erythroid RNA in each sample. The constant mouse β^{maj} message levels demonstrated that transgene expression does not affect the transcription of the endogenous mouse β^{maj} gene. The copy number data suggested that the founder mice were mosaic, and this was supported by the percentage of progeny that were transgenic (25, 28, 18, 28 and 20% for the β^A E lines). Measurement of the

TABLE 1 Transgene DNA copy number and RNA abundance

Line	Founder		Progeny	
	DNA copy number	RNA abundance	DNA copy number	RNA abundance
β^A E				
2671	1.7	2.1	3.2 ± 0.5 (3)	3.1 ± 0.2 (3)
2673	2.2	6.3	6.5 ± 1.0 (11)	8.3 ± 1.6 (6)
2674	1.5	1.6		
2680	30		37 ± 7.6 (5)	61 ± 21 (4)
2681	5.7	7.1	7.2 ± 0.1 (2)	11.9 ± 0.7 (4)
2702	1.8	1.4	7.7 ± 0.5 (2)	8.3 ± 2.1 (3)
β^A ΔE				
2396(A+B+C)	72	<0.2		
2396(A)			28 ± 12 (6)	<0.02 (3)
2396(B)			46 ± 17 (3)	<0.02 (1)
2396(C)			63 ± 7 (2)	<0.02 (2)
2399	10	<0.02	9.8 ± 0.1 (3)	<0.02 (2)
2404	0.3	<0.02	0.9 ± 0.1 (7)	<0.02 (3)
9569	1	<0.02		
9581	11	<0.02	24 ± 10 (2)	<0.02 (2)

DNA copy number is presented in copies per diploid genome. β^A RNA abundance is expressed as percentage of mouse β^{maj} RNA, uncorrected for either β^A or β^{maj} gene copy number. Numbers are mean ± s.d. (number of independent observations). Independent observations are defined as different animals, except for pairs of RNA measurements on the same animals bled at different times. Founder 2396 had three separate integration sites, denoted A, B, and C.

DNA copy number was measured by slot blotting²⁴. Tail DNA (3 μ g) was bound to GeneScreen Plus, in duplicate, and detected by hybridization with probe 1142. Linearly exposed autoradiograms were scanned and copy number was calculated from standards on the same blot by linear interpolation. Correct slot loading was confirmed by rehybridizing the blot to a glyceraldehyde 3-phosphate dehydrogenase cDNA probe²⁵. The actual copy number is integral in the progeny mice without any rearrangements. RNA abundance in blood was measured by primer extension (Fig. 2). The β^A , α , and β^{maj} bands were scanned, and integrated, and β^A was normalized to the mouse globins in the same sample. From the specific activities of the primers, β^A RNA abundance as a percentage of mouse β^{maj} RNA was calculated.

DNA copy number and RNA abundance in progeny mice eliminated the confounding effects of mosaicism. Figure 3*a* shows a plot of β^A RNA abundance per copy number against copy number. It is striking that over a 25-fold range of copy number, the RNA:DNA ratio is constant, exhibiting less than twofold variation.

These data provide evidence that transgene expression is position-independent. Proof of position independence requires only that all lines carrying the same number of gene copies express the same amount of mRNA. That the abundance of β^A RNA per copy number is the same over a 25-fold range of copy number suggests in addition that all copies of the integrated genes are expressed equally. We can compare the coefficient of variation (CV) of the RNA:DNA ratios shown in Fig. 3*a* with that obtained for the human β -globin gene: the CV is 23% for the chicken gene, comparable to the value of 12% for the human β -globin gene with upstream elements. In contrast, the CV is 130% without these elements (calculated respectively from ref. 5 and ref. 13, -815h β G construct).

The relatively low level of β^A RNA (2.7% that of mouse β^{maj} , per copy) is perhaps not surprising, as far upstream hypersensitive sites¹⁴ (which include enhancers (unpublished observations, see ref. 15)) which might augment the local elements, were not included in the β^A E fragment. It is also possible that in the 250 million years since the common ancestor of chickens and mice, divergence of *cis* elements or *trans* factors may have occurred (see ref. 16 for an example), resulting in less efficient expression of the heterologous gene. Dissociation of

position independence from high level expression also occurs in the human β -globin locus¹⁷. Irrespective of the level of β^A RNA, it is clear that the chicken elements mediating position-independent expression can function in mice. Thus the mechanisms and mediators responsible for expression independent of integration site arose before the divergence of Mammalia and Aves and have not changed significantly since then. The β^A transgenic mice also express β^A -globin protein in a copy-number dependent manner (Fig. 3b).

This strict dependence on copy number, together with the observation that every transgenic mouse expresses the chicken β^A -globin gene, is evidence that the β^A E fragment carries the information necessary for position independence. We do not know which regions within β^A E cause position independence, but the β^A/ϵ enhancer is nuclease-hypersensitive in chromatin and thus a likely candidate. It is interesting that both the chicken β^A E fragment and complete constructs of the human β -globin LCR contain the strongest enhancer of their respective clusters^{15,18}. Both the chicken β^A E and the larger, more effective versions of the human β LCR constructions contain matrix attachment regions (putative sites of DNA attachment to the nuclear matrix or scaffold)^{19,20}. The functional contribution of matrix attachment regions to LCR activity is, however, unclear.

The chicken globin element we describe is located in the midst of the gene cluster, whereas the elements of the human β -globin LCR are far upstream from the genes they regulate^{5,15,21-23}. The intracuster location demonstrates that position independence is not necessarily mediated by regions at the boundary of a gene cluster or chromatin domain.

Little is known about the mechanisms bestowing position-independent gene expression. For example, there is no evidence that the elements mediating this effect share a unique, defining sequence motif or bind a distinct protein. The ability of a relatively well understood part of the chicken β -globin locus to confer position independence bodes well for our ability to dissect the details of this phenomenon. □

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